Universidade de Aveiro Secção Autónoma de Ciências da Saúde
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Fosforilação anormal de proteínas na Doença de Alzheimer

Abnormal protein phosphorylation in Alzheimer's disease

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Ana Gabriela Henriques, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e co-orientação da Professora Doutora Odete da Cruz e Silva, Professora Auxiliar da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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palavras-chave

Doença de Alzheimer, proteína precursora da amilóide, Abeta, fosforilação de proteínas

resumo

A Doença de Alzheimer é uma patologia neurodegenerativa caracterizada pela presença de placas senis, emaranhados neurofibrilhares e perda sináptica. Aβ, o principal componente das placas senis, tem um papel fundamental na patologia, uma vez que, o aumento da sua produção e agregação está associada a neurotoxicidade, activação da resposta inflamatória e cascatas apoptóticas. Estes processos estão associados à morte neuronal, neurodegeneração e, consequentemente, com o declínio cognitivo gradual. Considera-se também alterações em vias de sinalização celular como um dos aspectos fundamentais da patologia. A fosforilação de proteínas é reconhecida como um mecanismo fundamental capaz de regular eventos intracelulares. Vários estudos têm descrito uma actividade anormal de proteínas cinases e fosfatases em cérebros de doentes, bem como níveis anormais de fosforilação da PPA (Proteína Precursora de Amilóide de Alzheimer), Tau, entre outras proteínas. A fosforilação é um dos mecanismos que regula as funções e processamento da PPA sendo esta uma proteína fosfo-específica descrita como hiperfosforilada nos cérebros de doentes de Alzheimer.
Devido à importância do Aβ e aos níveis de fosforilação anormal descritos na DA, os objectivos deste estudo eram a análise dos efeitos do Aβ na fosforilação da PPA, em específico nos resíduos Thr668 e Tyr682, bem como nos níveis de fosforilação geral. Neste trabalho observaram-se níveis aumentados de fosforilação nos resíduos especificados após a exposição ao Aβ sugerindo que este possa estar relacionado com a fosforilação anormal da PPA e, consequentemente, com o processamento desta. O trabalho desenvolvido sugere ainda o envolvimento da proteína fosfatase 1 na desfosforilação da PPA no resíduo Thr668. Adicionalmente, tanto o Aβ como a fosforilação no resíduo Thr668 regulam as interacções da PPA. Análise do fosfoproteoma revelou alterações em resposta ao tratamento com Aβ, estando proteínas envolvidas na transcrição de genes, cinases e fosfatases, aumentadas após o tratamento com Aβ. Todos estes resultados sugerem que o Aβ pode estar relacionado com a fosforilação anormal de proteínas conduzindo a uma sinalização intracelular anormal e consequentemente, contribuindo para a DA.

keywords Alzheimer´s disease, amyloid precursor protein, Abeta, protein phosphorylation

abstract

AD is a neurodegenerative disorder neuropathologically characterized by the presence of senile plaques, neurofibrillary tangles and synaptic loss. The Aβ peptide, the major constituent of senile plaques, is a key player in AD pathology since increased Aβ production and aggregation was associated with neurotoxicity, activation of inflammatory response, and apoptotic cascades. These processes are associated with neuronal death, neurodegeneration and consequently gradual cognitive decline. Altered signal transduction is also thought to be one of the key aspects in AD pathology. Protein phosphorylation is recognized as a fundamental mechanism by which the regulation of key intracellular events is achieved. Several studies have reported abnormal protein kinase and protein phosphatase activities in AD brains as well as abnormal phosphorylation levels of APP and Tau proteins. Further, phosphorylation is one of the mechanisms that regulates APP function and processing. APP is a phospho-specific protein also described as being hyperphosphorylated in AD brains.

Due to the key role played by Aβ and abnormal phosphorylation in AD pathology, the aim of this study was to analyze de Aβ effects on APP phosphorylation at Thr668 and Tyr682 as well on protein phosphorylation in general. In this work we could observe an increase in the phosphorylation level of APP at these specific residues upon Aβ exposure at low concentrations. The phosphatase involved in dephosphorylating the above mentioned residue (Thr668) was found to be protein phosphatase 1. Additionally, it was also observed that both Aβ and APP phosphorylation at Thr668 can regulate APP interactions. The phosphoproteome was in fact altered in response to Aβ exposure, and it was shown that proteins involved in fundamental cellular processes such as gene transcription and intracellular levels of protein kinases and phosphatases are increased upon Aβ treatment. Taken together these findings suggest that Aβ plays a role in abnormal protein phosphorylation, potentially leading to abnormal signaling cascades, and consequently contributing to AD pathology.

ABBREVIATIONS

Abl	Abl protein tyrosine kinase
AD	Alzheimer's disease
ADAM	A Disintegrin and Metalloproteinase
AICD	APP Intracellular Domain
Akt	Serine/threonine-protein kinase akt
APLP1/2	Amyloid Precursor Like Protein 1/2
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
APP-BP1	Amyloid Precursor Protein-Binding Protein 1
APS	Ammonium Persulfate
ARF	ADP-Ribosylation Factor
ARH	Autosomal Recessive hypercholesteremia
Aβ	Amyloide β-peptide
BACE	Beta-site APP Cleaving Enzyme
BCA	Bicinchonic Acid
BSA	Bovine Serum Albumin
Cant	Cantharidin
Cdc2	Cyclin-dependent kinase 2
Cdk5	Cyclin-dependent kinase 2
CRMP-2	Neurofibrillary Tangle-associated Collapsin Response Mediator Protein-2
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
CTF's	C-Terminal Fragments
CuBD	Copper-Binding Domain
Dab1/2	Disabled homolog 2-interacting protein
DTT	Dithiothreitol
Dyrk1A	Dual-specificity tyrosine-regulated kinase 1A
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid

ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
FAD	Familial Alzheimer´s disease
FBS	Fetal Bovine Serum
GFLD	Growth Factor Like Domain
Grb2	Growth factor receptor-bound protein 2
GSK-3β	Glycogen synthase kinase 3β
HBSS	Hanks Balaced Solution
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
$_{Holo}$APP	Total APP
HSPG	Heparin Sulphate Proteoglycan binding-site
IDE	Insulin Degrading Enzyme
IP	Immunoprecipitation
Jip 1b/2	C-Jun-amino-terminal kinase-interacting protein 1b/2
JNK3	C-Jun-amino-terminal kinase 3
KLC	Kinesin Light Chain
KPI	Kunitz Protease Inhibitor
LDL	Low Density Lipoproteins
LGB	Low Gel Buffer
LRP	Lipoprotein Receptor-related Protein
Lyn	Tyrosine-protein kinase Lyn
MAP kinase	Mitogen-Activated Protein Kinase
MARK	Microtubule-Affinity Regulating Kinase
MCI	Mild Cognitive Impairment
MDC	Metalloprotease/Disintegrin-like
MOPS	3-(N-morpholino)propanesulfonic acid
MRI	Magnetic Resonance Imaging
nACh receptors	Nicotinic acetylcholine receptors
NEP	Neprilysin
NFT	Neurofibrillary Tangles
NMDA	N-Methyl D-Aspartate receptor
NPXY motif	AsparagiNes-Proline-any-tYrosina
NSAID's	Non Sterois Anti-Inflammatory Drugs

Num	Nuclear migration protein
OA	Okadaic Acid
PAK3	Serine/threonine-protein kinase PAK 3
PAT-1	DNA Topoisomerase 2-Associated Protein PAT1
PBS	Phosphate Buffer Salt
PET	Positron Emission Tomography
PHF	Paired Helical Filaments
PI3K	Phosphoinositide 3-kinase
PID	Phosphotyrosine Interaction Domain
PK A/B/C	Protein Kinases A/B/C
PMSF	Phenylmethanesulfonylfluoride
PP1/2A/2B/4/5/7	Protein Phosphatases 1/2A/2B/4/5/7
PS1	Presenilin-1
PS2	Presenilin-2
Rab 5/11	Ras related protein 5/11
RAGE	Receptor for Advanced Glycosylation Endproducts
RIPA	Radio-Immunoprecipitation Assay
ROCK1	Rho-associated protein kinase
sAPP	Secreted APP
sAPPα/β	Secreted APP after α-cleavage/β-cleavage
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec1/Munc18	Syntaxin binding proteins
SEC-R	Serpin-Enzyme Complex Receptor
Ser	Serine
Shc	SHC-transforming protein
SNARE	Soluble N-ethylmaleimide sensitive factor-atachment protein Receptor
SorLA	Sortilin-related receptor
SP	Senile Plaques
SR-A	Class A Scavenger Receptor
Src	Proto-oncogene tyrosine-protein kinase Src
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween

TGN	Trans-Golgi Network
Thr668	Threonine 668
TRIS	Tris(hydroxymethyl)aminomethane
TrkA	Trk system potassium uptake protein trkA
Tyr682	Tyrosine 682
UGB	Upper Gel Buffer
UV-DDB	Ultra-Violet damaged DNA
VAMP 5/7/8	Vesicle-associated membrane protein 5/7/8
VPS 5/17/26/29/35	Vacuolar Protein Sorting 5/17/26/29/35

INDEX

1. INTRODUCTION

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative multifactorial illness first described in 1906 by the German pathologist Alois Alzheimer. It's characterized by a progressive memory loss and other cognitive functions decline, with insidious onset and slow and irreversible progressive worsening. Personality changes, such as behavioral and emotional disturbances are also observed and contribute to professional, social and personal activities decline. Initial memory impairment converts over time to disorientation, speech abnormalities, hallucinations, apraxias and incontinence, among other signs. At the end stage of the disease, patients are completely dependent on others and pneumonia is the principal cause of death in these patients (Castellani et al. 2010). The clinical duration of the disease is 8-10 years (Bird 2010). The estimated number of affected individuals is 4-5 million in E.U.A., 7-8 million in Europe, 90.000 in Portugal and 24 million worldwide (Jakob-Roetne and Jacobsen 2009; http://www.alzheimerportugal.org). AD affects nearly 8.4% of persons with 85 years or older, representing a growing public health problem as life expectancy increases (Candores et al. 2010).

1.1.1 Neuropathological Hallmarks of AD

AD hallmarks are extracellular senile plaques (SP), intraneuronal neurofibrillary tangles (NFT) and neuropil threads (abnormal neurites) (Figure 1). These lesions are found in specific brain regions involved in memory and learning processes namely the neocórtex, entorhinal cortex and hippocampus. The presence and distribution of NFT, SP and synaptic degeneration correlate with the degree of cognitive decline (Shankar and Walsh 2009), and at the time of the first symptoms of episodic memory problems, pathological changes are already present in the brains of AD patients.

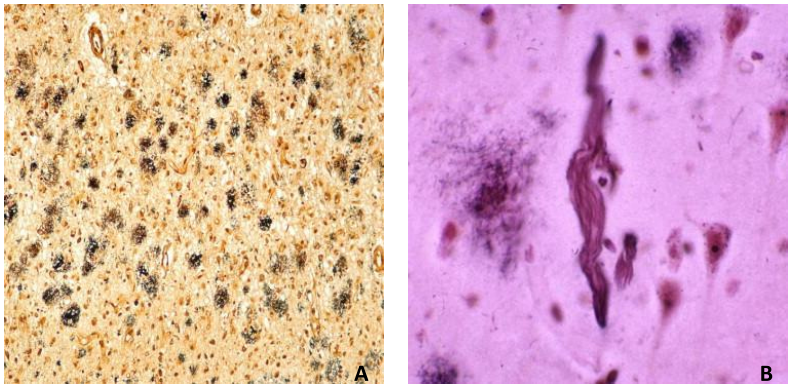

Figure 1. A. Senile Plaques **B.** Neurofibrillary tangles. Seen with Bielshowsky silver stain (Castellani et al. 2010)

Senile Plaques

Senile plaques (SP) (Figure 1 A) are extracellular deposits found in AD brains, mainly composed of amyloide β-peptide (Aβ) aggregates (Aβ derives from proteolytic cleavage of Amyloid Precursor Protein (APP), Section 1.2), found in AD brains but also in normal aging. SP are also observed in individuals with Mild Cognitive Impairment (MCI) at a higher level than normal older adults and it is a strong predictive factor in conversion to AD. The Aβ deposition spreads from regions with early on existing deposits into regions which receive their neuronal input. A strong correlation exists between the degrees of Aβ deposition and clinical symptoms. Indeed, several studies support the amyloid hypothesis which defends that Aβ accumulation in central nervous system is the first event that initiates the pathogenic cascade that culminates in neuronal death and neurodegeneration typical of AD (Figure 2).

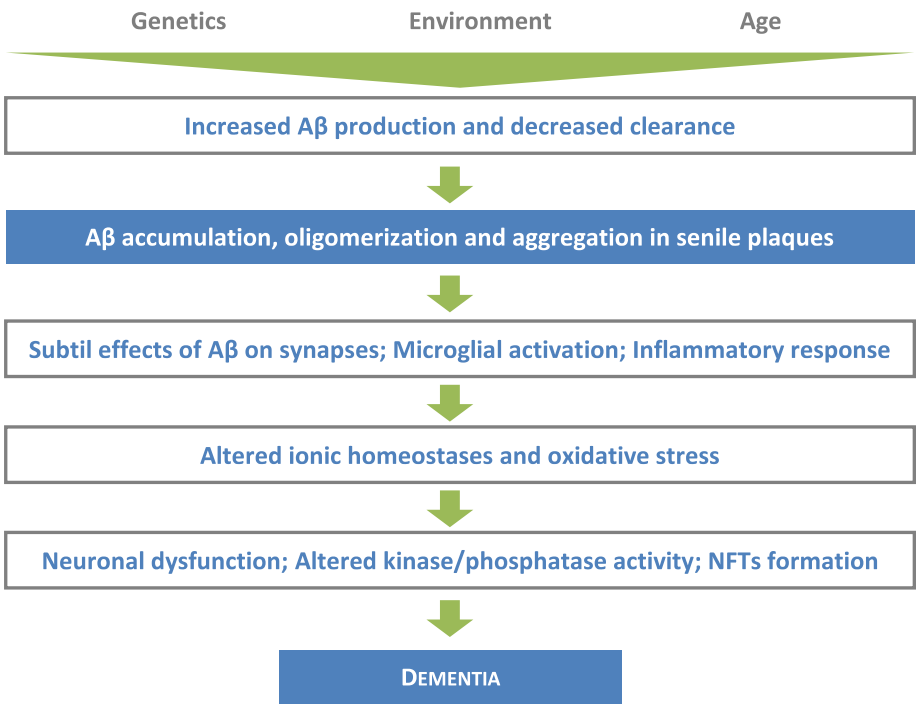

Figure 2. Schematic representation of the amyloid cascade hypothesis of AD. Adapted from Jakob-Roetne and Jacobsen 2009 and http://www.alzforum.org/res/adh/cur/knowntheamyloidcascade.asp.

Accordingly to this hypothesis, genetics, age and environment factors lead to an imbalance of Aβ production (from proteolytic cleavage of APP) and clearance. Aβ accumulation favours the oligomerization and aggregation processes and amyloid deposition, being these events related

with the histopathological and clinical manifestations of the disease (Jakob-Roetne and Jacobsen 2009).

The nonfibrillar $A\beta_{1-42}$ form is the predominant form found in diffuse plaques that leads to reactive astrocytes which will prevent the ability of microglia cells to remove amyloid material. Astrocytes in contact with diffuse plaques were shown to accumulate cell debris, from degenerated synapses and dendrites, besides $A\beta_{1-42}$. Microglia activation in AD brains had been involved in Aβ degradation by phagocytosis and this phagocytic activity is responsible for the production and release of H_2O_2 and pro-inflammatory cytokines and chronic microgliosis. These alterations will contribute to neuronal dysfunction and death and therefore to disease progression (Jakob-Roetne and Jacobsen 2009).

- **Neurofibrillary tangles**

NFT and neuropil threads consist in aggregates of abnormal paired helical filaments (PHF) of hyperphosphorylated Tau protein (Figure 1 B). Tau is a microtubule-associated protein involved in crucial neuronal process, such as microtubule dynamics, neurite outgrowth and axonal transport, and is regulated in a phosphorylation dependent manner. In its hyperphosphorylated state, Tau can sequester normal Tau and others microtubule-associated proteins, destabilizing and depolymerising the microtubules. As consequence, axonal transport and neurotransmission are compromised, being the more vulnerable regions the distal sites with synapses, resulting in a decline of cognitive functions. Moreover, hyperphosphorylated Tau self assembly lead to small deposits (pretangles) that adopt a β-sheet conformation in PHF. These in turn assemblies into large NFT in which Tau undergo additional modifications, namely, truncations, glycations and cross-linking by transglutaminases (Jakob-Roetne and Jacobsen 2009; Metcalfe and Figueiredo-Pereira 2010). Therefore, alteration in signalling cascades that lead to abnormal protein phosphorylation or aggregation can potentially contribute to NFT formation and neuronal degeneration.

1.1.2 Genetic Factors

Despite of the majority of AD cases are sporadic, rare, familial, early-onset autosomal dominant forms of AD (FAD) also exists. Mutations or polymorphisms in genes encoding APP, PS1 (Presenilin-1) and PS2 (Presenilin-2) (Lazarov and Marr 2010) are related to FAD.

Although disease-causing mutations in APP are rare, more than 25 mutations in *APP* gene are related with FAD and suggest that the disease can be initiated due to abnormal APP processing. All the mutations occur in the flanking region of Aβ domain. Duplication of *APP* gene is also responsible for the early-onset of the disease explaining why individuals with trisomy 21 have the increased risk of develop AD (Thinakaran et al. 2008). Missense mutations in APP represents less than 0.1% all the AD cases (Selkoe 2001).

Missense mutations in *Presenilin 1* (*PS1*, chromosome 14) and *Presenilin 2* (*PS2*, chromosome 1) genes are related to an early (between 40 and 60 years) and aggressive form of AD. Some studies reveal that mutations in these two genes shift the substrate specificity of PS1 and PS2 from Notch to APP, resulting in an increased production of Aβ (Ling et al. 2003).

Additionally, the presence of two alleles ApoE4 is one of the most important genetic risk factor to AD. The presence of ApoE4 precipitates the onset of the disease. In the other hand, ApoE2 seems to have a protective effect against de disease (Ehehal et al. 2003).

Regarding the non genetic factors, a Health and Aging Canadian Study made prospective study and demonstrate that arthritis, regular use of NSAID's, wine and coffee consumption, and regular physical activity were associated with a lower risk for developing AD. On the other hand, aging and low educational levels were associated with increased risk of AD incidence (Lindsay et al. 2002). Elevated serum levels of cholesterol and LDL are related to increased amounts of Aβ in the brain and subsequently increase the risk of developing AD (Ehehal et al. 2003).

1.1.3 Diagnosis of AD

The criteria for the diagnosis of dementia require the presence of multiple cognitive deficits in addition to memory impairment. The diagnostic approach for AD begins with clinical history, physical examination and cognitive testing. At this phase there is a clinical recognition of a progressive memory decline, a decrease in patient's ability to perform daily living activities, psychiatric problems, personality changes and behavioural problems. During the diagnostic process, interviewing friends and family may be helpful (Santacruz and Swagerty 2001).

Neuroimaging techniques are also used as tools for AD diagnosis, namely MRI (magnetic resonance imaging), CT (computerized tomography) and PET (positron emission tomography). At MRI, structural brain changes can be visualized due to substantial neuronal loss. MRI exams are routinely requested and are helpful to rule out other possible causes of dementia as brain tumor and vascular lesions (Mosconi et al. 2007). PET assesses the Aβ deposition in brain using a radioactive compound named S. Pittsburg compound which binds specifically to Aβ and does not bind to neurofibrillary tangles or Lewy Bodies at the concentrations achieved during PET scan (Fripp et al. 2008). Nonetheless, diagnostic confirmation is based on post-mortem observation of the specific pathological lesions like NFT, SP and synapse dysfunction and loss (Mosconi et al. 2007).

1.1.4 AD Treatment

Similar to others neurodegenerative diseases, therapy for AD is based on transmitter-replacement therapies using compounds that increase acetylcholine levels in the brain, facilitating cholinergic neurotransmission through inhibition of acetilcholinesterases. Some examples of cholinesterases inhibitors are: donepezil, rivastigmine and galantemine. However, only a modest symptomatic benefit on cognitive, behavioral and global measures was seen in clinical trials of AD patients treated with these drugs. Other example of transmitter-replacement therapies is the antiglutaminergic treatment. Memantine, an NMDA antagonist, blocks excessive glutaminergic transmission that may lead to excitotoxity due to high intracellular calcium concentration in cases of overstimulation. Some studies support that this drug only has symptomatic rather than disease-modifying benefits (Lleó et al. 2006).

Concerning the behavioral and emotional disturbances, antipsychotics, antidepressants and anxiolytics, may be useful in the management of the disease. Nonpharmacological interventions

(music, light exercise, physical exercise, and relaxation) should also be considered (Lleó et al. 2006).

Nowadays, growing evidences support that oxidative stress and inflammation play an important role in AD. These conclusions offer a new therapeutic strategy focusing on anti-oxidant and anti-inflammatory drugs (Lleó et al. 2006).

Furthermore, as the knowledge about pathological mechanisms underlying AD, new therapeutic approaches emerge. The second generation of drugs to AD treatment is based on the amyloidogenic hypothesis, targeting different steps of the amyloidogenic pathway with various immunotherapeutic approaches. The drugs on clinical trials are inhibitors or modulators of secretases, inhibitors of amyloid aggregation and compounds that increase the clearance of Aβ. It is expected that these disease-modifying agents are able to retard disease progression (Jakob-Roetne and Jacobsen 2009).

1.2 Alzheimer´s Amyloid Precursor Protein (APP)

1.2.1 Gene and Protein Structure

APP is one of three members of a small gene family, which includes APLP1 and APLP2 (human) but only APP contains sequence encoding Aβ domain (Thinakaran et al. 2008). It is a ubiquitously expressed type I membrane glycoprotein that is encoded by a single gene on chromosome 21q21 and multiple isoforms exist resulting from alternative splicing of exons 7, 8 and 15 of the APP mRNA. The predominant transcripts are APP_{695}, APP_{751} and APP_{770}. The 695 isoform is the only one that lacks a kunitz protease inhibitor (KPI) domain in its extracellular portion. Although the functional relevance of this domain is unclear, KPI-containing isoforms of APP in human platelets were shown to serve as inhibitors of factor XI in the coagulation cascade (Selkoe 2001). APP is expressed in many cells and tissue types including endothelia, glia and neurons. Whereas APP_{695} is the predominant isoform in neuronal cells, APP_{751} and APP_{770} are predominantly expressed in peripheral tissues.

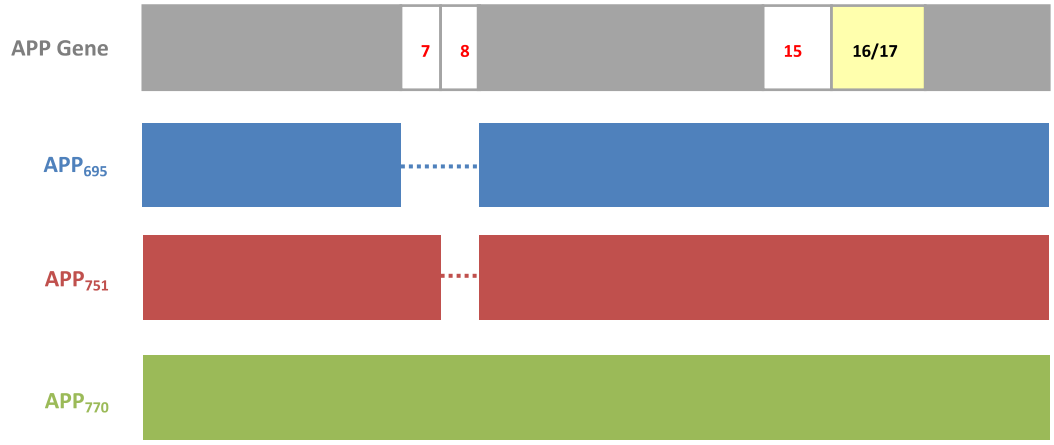

Figure 3. Schematic representation of APP gene and the three major APP isoforms. In red are the exons alternatively spliced. Aβ derives from parts of exons 16 and 17. The relative sizes of exons are not draw to scale (based on Ling et al. 2003).

Regarding the structure, APP has a single transmembrane span that separates the large N-terminal extracellular domain from the short C-terminal tail. The extracellular domain has a complex structure, having more than 70% of the residues. In this domain we can found two main domains: E1 and carbohydrate that are linked by an acidic region. E1 domain is composed by the growth factor-like domain (GFLD) and the copper-binding domain (CuBD). The carbohydrate

domain can be divided in the E2 domain, which contains the RERMS sequence, and the linker region.

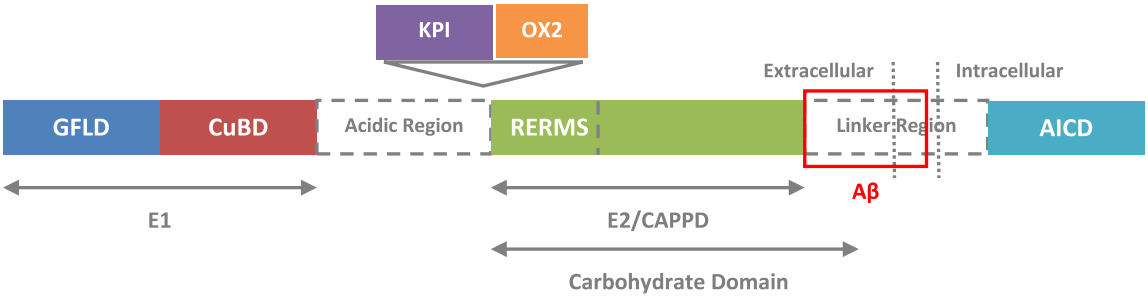

Figure 4. Schematic representation of APP domains. E1 region includes de N-terminal growth factor like domain (GFLD) and the copper-binding domain. The carbohydrate domain can be divided in E2 domain (also called central APP domain – CAPPD) and the linker domain. KPI (present only in APP_{751} and APP_{770} isoform) and OX2 sequence (present in APP_{770} isoform) are shown above their insertion site. The transmembrane domain and APP intracellular domain (AICD) are in the C-terminus. In red is represented the Aβ region.

Growth factor-like domain (GFLD) is a cysteine rich domain with three disulfide bridges that form a hydrophobic core. This domain seems to be important for neurite outgrowth and MAP kinase activation. The hydrophobic core is important for protein-protein interaction and it can be a possible interface for APP-APP dimerization. The possibility that this is a ligand-binding site should also be considered (Reinhard et al. 2005).

Copper-binding domain (CuBD) is similar among the different APP family parologs and orthologs, suggesting conservation in its function and activity. CuBD can modulate Cu^{+} mediated neurotoxicity and promote or inhibit copper neurotoxicity. Interaction between APP-Cu^{+} and H_2O_2 results in Cu^{+} oxidation to Cu^{2+} and APP fragmentation (Barnham et al. 2003).

In the carbohydrate domain, the CAPPD domain had possible interactions sites for binding partners and the RERMS sequence is implicated in the growth promoting properties of APP. In addition, E2 domain contains the highly conserved heparin sulphate proteoglycan-binding site (HSPG). E2 domain is able to form an antiparallel dimer in solution, consequently dimerization and dissociation of APP might regulate various aspects of APP functions (Reinhard et al. 2005).

APP intracellular domain (AICD) contains at least three functionally important motifs enabling APP interaction with several binding-partners (Figure 5 and Table 2 in APP-Binding Proteins section). These protein interactions may regulate APP/AICD function, localization and processing. The highly conserved 682-YENPTY-687 motif is recognized by proteins containing phosphotyrosine interaction domains (PID), namely, Fe65 family, c-Jun N-terminal kinase interacting protein, X11 family, Shc family and is also important to clathrin-mediated endocytosis. To the 667-VTPEER-672

motif binds the 14-3-3γ protein that is highly expressed in brain, skeletal muscle and heart. On the other hand, PAT1, a microtube interacting protein, binds to the 653-YTSI-656 motif (Slomnicki and Lesniak 2008). AICD has an unstable conformation in solution and the interaction with a binding-partner will stabilize its structure. This property is called binding promiscuity or "one-to-many" signalling. The stability of AICD-ligand complex can also be influenced by AICD phosphorylation, namely in Thr668 residue (Reinhard et al. 2005).

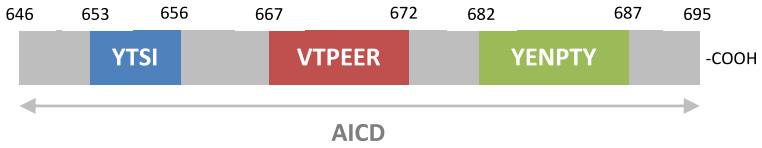

Figure 5. Representation of the AICD motifs.

1.2.2 APP Processing

APP can undergo distinct processing pathways: the non-amyloidogenic and the amyloidogenic pathway (Figure 6). In the first one, APP is cleaved by α-secretase within the Aβ domain resulting in the shedding of nearly the entire ectodomain (releasing a secreted APP fragment, sAPP) and generation of a membrane anchored α-C-terminal fragment (C83). The later is cleaved by γ-secretase complex releasing a non-toxic p3 peptide and the AICD polypeptide fragment. In the amyloidogenic pathway, APP is cleaved by β-secretase, releasing sAPPβ and β-C-terminal fragment (C99). Subsequently this fragment is cleaved by γ-secretase complex giving rise to Aβ peptide and the AICD fragment.

Cleavage of APP by activation of α-secretase is the relatively major and ubiquitous pathway of APP metabolism in most cells and leads to a significant decrease in Aβ formation. Estrogen, testosterone, various neurotransmitters, growth factor and protein kinase C (PKC) are able to regulate de α-cleavage pathway (Ling et al. 2003). Zinc metalloproteases like TACE/ADAM17, ADAM9, ADAM10 and MDC-9 can cleave APP at α-secretase site (Thinakaran et al. 2008).

In the amyloidogenic pathway, particularly enriched in neurons, APP is first cleaved by β-secretase, a transmembrane aspartyl protease. BACE1 and BACE2 are two enzymes capable of cleavage at β-site, being the first one the major β-secretase in the brain and is the key rate-limiting enzyme that initiates the Aβ formation. Over-expression of BACE1 in cell culture had been shown to increase the amount of β-secretase cleavage products. BACE2 shows similar substrate specificity but is not highly expressed in the brain (Ling et al. 2003).

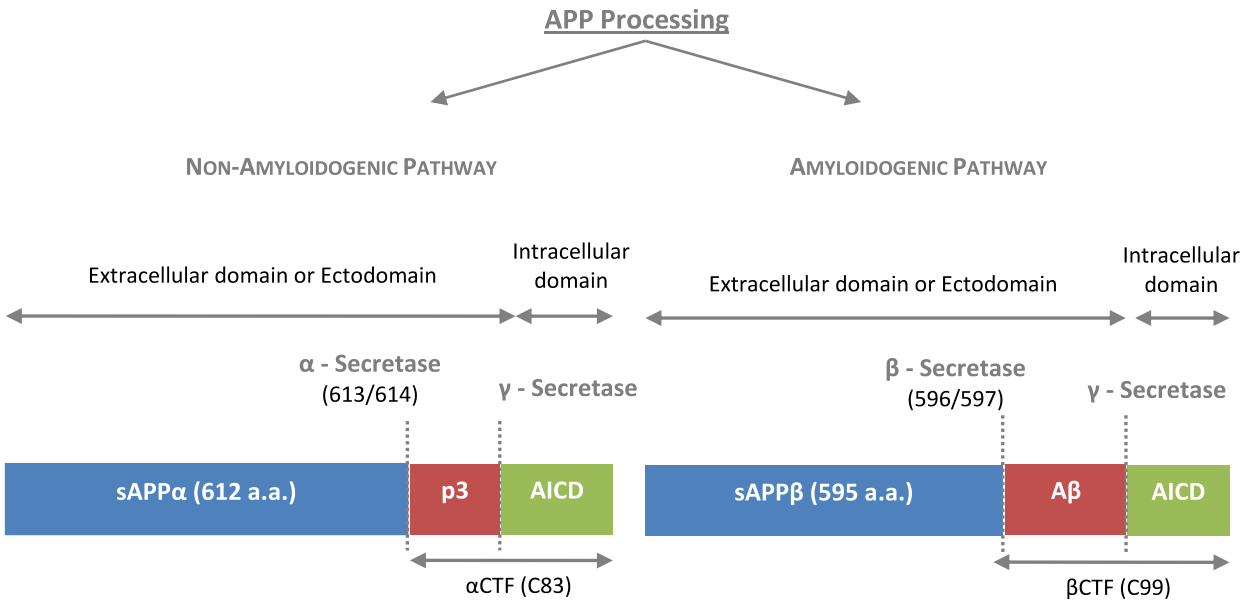

Figure 6. APP processing pathways. Non-amyloidogenic pathway occurs mainly at the cell surface. APP is cleaved by α-secretase between the residues 613-614 (695 isoform numbering) originating the sAPPα that is secreted to the extracellular medium and the CTF C83 that remains attached to the plasma membrane. C83 is cleaved by γ-secretase originating the p3 peptide and the AICD fragment. **Amyloidogenic pathway** occurs manly in endosomes and trans-Golgi network. APP is cleaved by β-secretase, between the 596-597 residues, originating sAPPβ that is secreted to extracellular medium as sAPPα. The CTF C99 is subsequently cleaved by γ-secretase complex originating Aβ peptide and AICD fragment (Adapted from Turner et al. 2003).

γ-Secretase is a complex entity composed by four subunits: Presenilin-1 or 2 (PS1 and PS2), nicastrin, APH and PEN-2. PS1 and PS2 are ubiquitously expressed membrane proteins with eight membrane-spanning regions with cytosplasmic orientation for both the N- and C-terminus. The biological action mechanism of presenilin is unknown but evidences suggest that they are involved in a range of biological processes, namely, cell adhesion, G-protein mediated signalling and unfolded protein response signalling. Nicastrin, a type I transmembrane glycoprotein, that forms high molecular weight complexes with PS1 and PS2 that regulate each other and determine γ-secretase function and affects APP processing and trafficking (Ling et al. 2003). γ-Secretase complex cleaves at multiple sites within the transmembrane domain of APP, generating Aβ peptides ranging in length from 38 to 43 residues.

1.2.3 Intracellular APP Trafficking

Regarding cellular localization, APP can be found in many membranous structures in the cell, namely, endoplasmic reticulum, Golgi complex and cell membrane (Turner et al. 2003; Ling et al. 2003). It is synthesized in the endoplasmic reticulum (ER) and migrate to the plasma membrane where is rapidly internalized due to the presence of YENPTY internalization motif near the C-terminus of APP (residues 682-687 of the APP_{695} isoform). During its traffic to the plasma membrane, APP is post-translational modified by N- and O-glycosylation, ectodomain and cytoplasmic phosphorylation, and tyrosine sulfation. Only 10% of APP synthesized arrived the plasma membrane in the steady-state. At the cell surface, APP can suffer α-cleavage, initiating the non-amyloidogenic pathway, or it can be internalized to endosomes. Once in the endosomes, can be recycled to the cell surface, degraded in lysosomes or went to the amyloidogenic pathway, originating Aβ peptide (Figure 7). Amyloidogenic processing occurs in cholesterol and sphingolipid-enriched membrane raft microdomains of intracellular organelles, taking place mainly in endosomes and trans-Golgi network through the secretory and recycling pathway (Thinakaran et al. 2008).

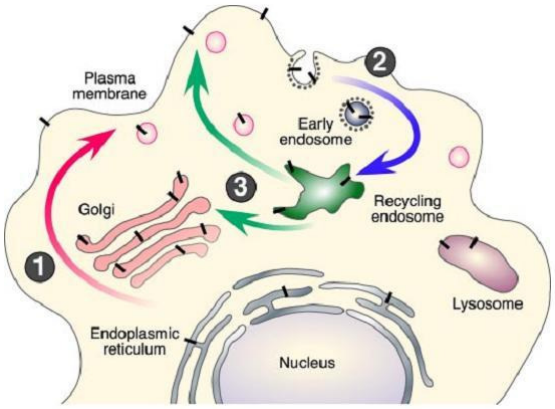

Figure 7. Intracellular APP trafficking. 1. Maturation of APP; during its traffic from ER to plasma membrane APP suffers post-translational modifications: N- and O-glycosylation, phosphorylation and tyrosine sulfation. **2.** Once in the plasma membrane, APP is internalized in endosomes. **3.** Once in the endosomes it can be recycled to the plasma membrane or degraded in lysosomes. (Reproduced from Thinakaran et al. 2008)

APP contains in it cytoplasmic tail the asparagiNes-Proline-any-tYrosina (NPXY) motif that targets to clathrin pit localization and enables it transport from the cell surface to the endosomes via clathrin-coated vesicles (Small and Gandy 2006).

The retromer is the second complex implicated in APP transport from the endosome to the TGN. Retromer is a multimeric complex highly expressed in the brain and composed by vacuolar protein sorting (VPS), namely VPS35 (the core component of the retromer complex), VPS26, VPS29, VPS5 and VPS17 (Small 2008).

The fusion of transport vesicle with the target organelle involves SNARE (soluble N-ethylmaleimide sensitive factor-attachment protein receptor), Rab (sub-family of the Ras superfamily of low-molecular-mass GTPases) and Sec1/Munc18 proteins. The specific combinations between these proteins guarantee the delivery of APP to the proper destination (Small and Gandy 2006).

APP is not the only protein transported via clathrin coated or retromer sorting pathway, BACE is also transported by these two systems. Various sorting molecules involved in both APP and BACE transport have been found and some of them are listed in Table 1.

Table 1. Molecules involved in APP and/or BACE sorting (Small and Gandy 2006).

Coat complexes	Clathrin, VPS35, VPS26A, VPS26B, VPS29, Sorting nexin 1, Sorting nexin 2, ARF
Coat adaptors	SorLA, Sortilin, ARH, Numb, Dab1,Dab2, Jip1b, Fe65, Fe65L1, Fe65L2, X11a, GGA1, GGA2, GGA3
Factors for docking and/or fusion	Syntaxin 5, 6, 7, 8, 13, 16; VAMP 5, 7, 8; Rab 5, 11; SM protein VPS45, Munc 13, 1
kinases, phosphatases	PKA, PKC, ERK, cdk5, GSK3, ROCK1, PP1, PP2A, PI3K, Akt, VPS30 (beclin)-VPS34-PI3K
Other factors	PS1, PS2, Nicastrin, APH1, PEN2, Lipid rafts, Caveolin, Flotillin, Cholesterol

1.2.4 Aβ PEPTIDE

Aβ is a polypeptide, of approximately 4 kDa, that is produced by proteolytic cleavage of APP. Various Aβ species exists with extensive amino and carboxyl terminal heterogenicity. Under physiological conditions $Aβ_{1-40}$ is the most common form (80-90%), followed by $Aβ_{1-42}$ (5-10%). Other peptides also exist but in lower amounts, for example, $Aβ_{1-38}$, $Aβ_{1-39}$ and $Aβ_{1-43}$ (Golde et al. 2000; Welander et al. 2009).

$Aβ_{1-40}$ is the major component of senile plaques that occur as clumps of insoluble amyloid fibrils (8-10 nm) mixed with the non fibrillar form and surrounding by distrophic neuritis. Fibril $Aβ_{1-42}$ is also found in senile plaques and in difuse plaques in its non fibrillar form. Aβ (mostly the 40-residue form) also accumulates within the basement membranes of cerebral capillaries, arterioles and venules and some meningeal arterioles (Selkoe 2001). Presence of Aβ is not exclusive of AD brains, cognitively normal human brains contains Aβ deposits, with relatively few SP. In 1992 was discovered that Aβ is constitutively secreted by healthy cells throughout life and is found in

cerebrospinal fluid (CSF) and plasma of all normal humans (Selkoe 2001). Deposited Aβ peptides can undergo several modifications, like pyrolization, oxidation, isomerisation and proteolytic degradation (Golde 2000). Although, Aβ peptides are found at extracellular level in difuse and senile plaques, it can also be found in the intracellular medium, nonetheless its function at this location is not completely understood.

Aβ has the ability to assembly and can exist in different assembly's forms, ranging from dimmers to aggregates of fibrils. $A\beta_{1-40}$ oligomers are composed by only three monomers, while $A\beta_{1-42}$ is able to form bigger oligomers (Lublin and Gandy 2010). In the aggregate form, Aβ seems to have a toxic gain function and to be able to interact with new structures, leading to impaired neuronal plasticity (Table 3) (Shankar and Walsh 2009). Nonetheless, recent studies defend that soluble Aβ oligomers may also play a key role in AD pathogenesis. Structurally, soluble Aβ40/42 peptides consist in two α-helices. In contrast, Aβ exhibit a β-sheet conformation in amyloid fibrils. The shift in the structural conformation might be important for the amyloidogenic properties of the peptide. Interestingly, disease-related mutants, like APP Dutch and Artic mutations within Aβ domain, cause structural destabilization, favouring the β-sheet conformation, and showing a high tendency of Aβ to aggregate (Reinhard et al. 2005).

Relative to Aβ degradation, two major enzymes are believed to be responsible for this process: neprilysin (NEP) and insulin degrading enzyme (IDE). Lysosomal degradation by enzymes such cathepsin B is also a possibility. NEP is a plasma membrane bound type II metalloproteinase responsible for extracellular degradation of a variety of peptides besides Aβ (e.g. glucagon, enkephalins, endothelin, substance P). It can degrade the insoluble form of Aβ but its effects on soluble form are less evident. IDE is also a metalloproteinase responsible for degradation of Aβ and insulin peptides that is active both intra and extracellularly. Lower levels of these two enzymes are observed in AD suggesting that low Aβ clearance is one of the causes to its accumulation in brain and CSF. Despite of the clearance mechanisms, an amount of Aβ remains undegraded and can cross the blood-brain-barrier into circulation, a process mediated by the lipoprotein receptor-related protein (LRP) and by the receptor for advanced glycation end products (RAGE) (Murphy and LeVine III 2010; Ling et al. 2003).

1.2.5 APP-Binding Proteins

APP binding-partners include serum proteins, cell surface proteins, extracellular matrix proteins, and signalling molecules. In Table 2 are summarized some of the APP-binding proteins. In most of the cases, the role of this interaction is unclear or unknown or not so clear, some of the roles presented for these interactions are only on the possibilities field.

Table 2. APP-Binding proteins and putative role of this interactions.

sAPPα	Heparin, Laminin, Collagen	APP binding to extracellular matrix, including, heparin, laminin and collagen, suggests a function in cell–cell and/or cell–ECM adhesion (Ando et al. 1999; Dahms et al. 2010)
	ApoE	Regulate sAPP clearance (Turner et al. 2003).
	Class A scavenger receptor (SR-A)	SR-A binds to sAPP and Aβ peptide and is thought to be involved in clearance of these two APP fragments. It also mediates microglia and human monocytes adhesion to amyloid fibrils and this process leads to secretion of reactive oxygen species (Santiago-García et al. 2003).
	Fibulin-1	Predominantly expressed in neurons and, together with sAPP, may regulate neural adhesion and neurite outgrowth (Turner et al. 2003).
Aβ	ApoE	ApoE removes secreted Aβ from extracellular medium and Aβ competes with lipids for ApoE, being involved in cholesterol up-take and homeostasis (Turner et al. 2003).
	RAGE	Receptor for advanced glycosylation endproducts is expresses in microglia and hippocampal neurons and is responsible for the removal and Aβ degradation (Turner, et al. 2003).
	SEC-R	Serpin-enzyme complex receptor internalizes and degrades soluble Aβ from the cytosol (Turner et al. 2003).
	nACh Receptors	One putative function of Aβ is the modulation of memory interaction. This modulation is thought to be achieved by Aβ interaction with nACh receptors (Garcia-Osta and Alberini 2009)
	PrPc	Cellular prion protein may act as neuronal receptor for oligomeric Aβ being responsible for 50% of neuronal oligomer binding capacity observed (Lublin and Gandy 2010).
AICD	Fe65 family Fe65, Fe65L1, Fe65L2	Fe65 form a ternary complex with APP and Tip60 that activate gene transcription. It had also been reported to influence cell motility (Wolf and Guénette 2007). Interaction between Fe65 and AICD is regulated by the phosphorylation state of AICD at Thr668 residue (Buoso et al. 2010).
	X 11 Family X11 (Mint1), X11L (Mint2)	X11 is neuronal cytosolic adaptor protein involved in basolateral protein sorting being involved in vesicle exocytosis (Buoso et al. 2010; Wolf and Guénette 2007). Prolongs APP half-life and inhibits Aβ generation by retarding exocytosis (Slomnicki and Lesniak 2008).
	JIP Family JIP-1b, JIP-2	Scaffold protein that binds to kinesin light chain 1 and have been implicated in different signalling pathways, including neuronal apoptosis. Expression of

AICD		JIP-1b stabilizes immature APP and modulates APP metabolism. JIPs are able to phosphorylate APP at Thr668 residue by activation of JNK (Buoso et al. 2010).
	F-spondin	Its signalling glycoprotein that may function in neuronal development and repair and regulates APP processing. The binding of F-spondin to APP reduces β-secretase cleavage of APP and nuclear transactivation of AICD (Thinakaran et al. 2008).
	G_0, PAK3, APP-BP1	Regulate cell cycle progression (Wolf and Guénette 2007).
	Pat1a	A microtube-interacting protein that plays a role in anterograde transport of APP and APLPs (Wolf and Guénette 2007).
	14-3-3γ	Protein kinase inhibitor reported to interact with AICD at 667-VTPEER-672 motif (Buoso et al. 2010).
	Shc family Shc A, Sch C	Shc family proteins generally connect growth factor receptors to specific signalling pathways. AICD phosphorylation at the tyrosine residue works as docking site to Shc A binding (Buoso et al. 2010).
	mDab1	MDab1 is an adaptor protein that participates in nervous system development. Is phosphorylated during embryogenesis and when in the phosphorylated state is able to increase cellular levels of mature APP and inhibit APP endocytosis (Buoso et al. 2010).
	Kinesin Light Chain (KLC)	Kinesin is a microtubule-based motility protein that mediates axonal transport and other intracellular movements. APP can be a receptor for KLC and its transport is mediated by this binding (Dhaenens et al. 2004).
	Num and Numb-like proteins	Numb is a negative regulator of Notch signalling pathway. Interaction between APP and Numb may be the cross-talk between APP and Notch signalling. Numb binding to APP may increase Notch activation (Merdes et al. 2004)
	Abl-non-receptor tyrosine kinase	Abl family non-receptor tyrosine kinases modulate short-term synaptic plasticity and cell morphogenesis. Abl interacts with YENPTY motif being related with AICD phosphorylation at Tyr682 residue (Moresco et al. 2002; da Cruz e Silva et al. 2004).
	ARH adaptor protein	ARH (autosomal recessive hypercholesteremia) adaptor protein is involved in LDL receptors internalization (Wolf and Guénette 2007). It interacts with AICD regulating cell membrane APP levels. Decreased levels of ARH adaptor protein leads to increased levels of APP. It postulated that ARH protein may be a link between cholesterol metabolism and APP processing (Noviello et al. 2003).
	UV-damaged DNA binding protein (UV-DDB)	It is known that UV-DDB interacts with AICD but the exact function of this interaction is unknown, however UV-DDB overexpression increase sAPP release. This protein was initially identified as a factor that binds to UV-damaged DNA (Watanabe et al. 1999).

1.2.6 APP AND ITS FRAGMENTS FUNCTIONS

Different functions have been attributed to the APP cleaved fragments, with $_{Holo}$APP, sAPP, Aβ and AICD being implicated in cell signalling and in a series of physiological processes. Many of the

proposed functions for APP and its fragments are related to biological functions likely neurite outgrowth, dendritic arborisation, synaptogenesis and synapse remodelling (Reinhard et al. 2005; Slomnicki and Lesniak 2008). Table 3 summarizes the functions for the different APP fragments as well as for $_{Holo}$APP.

Table 3. Putative functions of APP and its fragments.

	Functions	References
APP	▪ Synaptogenesis ▪ Synapse remodelling ▪ Neurite outgrowth ▪ Neuronal maturation and differentitiation ▪ Cell adhesion ▪ Cell surface receptor	▪ Thinakaran et al. 2008 ▪ Turner et al. 2003 ▪ Wolf and Guénette 2007
sAPPα	▪ Neuronal activation ▪ Neuroprotection ▪ Axonal and dendritic outgrowth ▪ Calcium homeostasis ▪ Membrane excitability regulation	▪ Turner 2003 ▪ Wolf and Guénette 2007
Aβ	**Physiological Functions** ▪ Cell adhesion ▪ Inhibition of cellular production and secretion of ApoE ▪ Cholesterol uptake and homeostasis ▪ Learning and memory ▪ Synapse Function ▪ Homeostatic plasticity ▪ Neuronal survival **Pathological functions** ▪ Formation of toxic oligomers ▪ Apoptotic properties ▪ Difuse and Senile plaques ▪ Impaired neuronal plasticity ▪ Inflammatory response ▪ Imbalanced protein kinase/phosphatise activity	▪ Lublin and Gandy 2010 ▪ Shankar and Walsh 2009 ▪ Golde et al. 2000
AICD	▪ Transcription activation ▪ Neurite outgrowth ▪ Genesis and/or synapse remodelling	▪ Kimberly, W. T., et al (2005) ▪ Buoso, E., et al (2010)

1.3 Protein Phosphorylation in Alzheimer's disease

Protein phosphorylation is the major post-translational modification mechanism, participating in a number of regulatory processes and signalling pathways. From the functional point of view, this mechanism can affect key functions of proteins including their activity, interaction with other protein or protein sub-cellular localization (Rossignol 2006). Protein phosphorylation and dephosphorylation is mediated by protein kinases (PK) and protein phosphatases (PP), respectively. Abnormal protein kinase or phosphatase activity is related to several pathological conditions by altering the phosphorylation of critical proteins in normal cellular processes.

1.3.1 Abnormal protein Phosphorylation in AD brains

Aberrant phosphorylation is linked to AD pathogenesis. In fact, a lot of evidences came out showing that PK and PP activity alterations are evident in AD brains (Table 4). Generally, in AD brains, an increase in PK activity and a decrease in PP activity can be observed (Chung 2009).

Table 4. Protein kinases and phosphatases whose expression and/or activity are altered in AD brains (Adapted from Chung 2009).

Protein Kinases		Protein Phosphatases	
GSK3β	↑	PP1	↓
P25/Cdk5	↑	PP2A	↓
Dyrk1A	↑	PP5	↓
ERK1/2	↑	PP2B	↑
JNK	↑	Cdc25A	↑
P38	↑	Cdc25B	↑
CK1	↑	PTEN	↓
Ak/PKB	↑		
PKC	↓		
PKA	↓		

Alterations in PK and PP activities may be due to Aβ peptide. In fact, by that in the majority of the cases, Aβ have a positive effect on PK activity. In the case of GSK-3β, exposure of neurons to Aβ increases GSK-3β activity through the inhibition of PI3-kinase signalling (Hooper et al. 2008; Resende et al. 2008). GSK3β is a proline-directed serine/threonine kinase with functions in various cellular processes including, signalling pathways, metabolic control, apoptosis/cell survival and

oncogenesis, being also intimately involved in tau hyperphosphorylation, memory impairment, increased Aβ production and inflammatory response.

Another PK, Cdk5, involved in neurogenesis regulation, actin dynamics, axon guidance, membrane transport, dopamine signalling and Tau hyperphosphorylation is also influenced by Aβ. Town et al. demonstrate that, at least, soluble Aβ is a potent activator of p25/Cdk5 pathway, resulting in promotion of Tau phosphorylation "*in vitro*". Increased Cdk5 activity is also associated with increased Aβ levels and neurodegeneration, and these effects are thought to be mediated by phosphorylation of Tau, APP and PS1 (Chung 2009). Similar, Dyrk1A is also associated with Tau hyperphosphorylation and increased Aβ levels (Chung 2009). Another kinase involved in APP phosphorylation is c-Abl. It has been reported that Aβ induced c-Abl activity in hippocampal neurons and that inhibition decreases Aβ-induced neuronal apoptosis (Alvarez et al. 2004). A relation between Aβ and Src kinases family was also reported, since an increase in tyrosine phosphorylation was observed in response to Aβ treatment and this effect was reversed when Src inhibitors were added (Williamson et al. 2002).

Contrary to the PK mentioned above, PKA and PKB are decreased in AD brains. PKC have been shown to regulate α- and β-cleavage of APP being decreased levels of PKC associated with increased levels of Aβ (Wang et al. 2008).

Relative to the other players in the phosphorylation process, the PP's, PP1 and PP2A, play a major role in protein dephosphorylation and in AD pathogenesis (Chung 2009). PP1 belongs to the serine/threonine phosphatase family and is widely expressed and highly regulated. PP1 is involved in long-term depression, synaptic plasticity and APP secretion modulation. It was reported that Aβ inhibits PP1 activity potentially affecting its own production and other key phospho-dependent protein in AD (Vintém et al. 2009). PP1 inhibition stimulates sAPP secretion (da Cruz e Silva et al. 1995). PP2A is one of the most important serine/threonine phosphatases in mammalian brains, with important roles in cell growth, development and transformation (Liu and Wang 2009), and is a key PP in AD pathogenesis. It has been suggested that PP2A is involved in Aβ production and increased sAPP release (Liu and Wang 2009). Both PP were shown to be decrease in AD and to be involved in Tau hyperphosphorylation. Indeed, the latter protein is a good example of the consequences of an impaired balance between PK and PP. Thirty nine phosphorylation sites have been attributed to Tau and GSK3β, Cdk5, Dyrk1A, PKA and MARK are the PK responsible for its phosphorylation. In contrast, both PP2A and to a less extent PP1 are involved in Tau dephosphorylation, and as referred previously, their activity is down-regulated in AD brains. Other proteins have been referred to be hyperphosphorylated in AD brains, including dynein, CRMP-2

(Neurofibrillary Tangle-Associated Collapsin Response Mediator Protein-2), neurofilament heavy and medium subunits, β-tubulin and presenilins (Vijayan et al. 2001; da Cruz e Silva et al. 2002).

1.3.2 APP PHOSPHORYLATION AND AD PATHOGENESIS

Protein phosphorylation is one of the mechanisms responsible for the regulation of APP function, processing, subcellular localization and protein-protein interaction. As an integral membrane protein, APP can be phosphorylated both in the extra and intracellular domains. When mature APP is phosphorylated, its subcellular localization varies, being largely localized in plasma membrane of cell bodies and neuritis of mature neurons (Ando et al. 1999). PKC seems to have a central role in APP phosphorylation because it regulates APP cleavage by affecting the intracellular trafficking events. PKC prolongs the time spent by APP at the cell surface, favoring the α-secretase activity (da Cruz e Silva and da Cruz e Silva 2003). Therefore, reduced PKC activity in AD brains can contribute to amyloidogenic processing of APP and consequently to increased Aβ production.

At the ectodomain, two phosphorylation sites are known to be phosphorylated. Phosphorylation at Ser189 and Ser206, occurring mainly in Golgi secretory compartment and at cell surface, seems to be both phosphorylated in vivo (da Cruz e Silva et al. 2004). The AICD presents eight potential phosphorylation sites (Figure 8), seven of which were found to be phosphorylated in AD brains (Slomnicki and Lesniak 2008). The phosphorylation of these residues interfere with AICD function and, consequently, with APP function (Slomnicki and Lesniak 2008). Constitutive phosphorylation of AICD is neuron specific and is the only difference between the neuronal and non-neuronal APP (Ando et al. 1999).

Figure 8. AICD potential phosphorylation sites (Adapted from Slomnicki and Lesniak 2008) .

Particularly, increased APP phosphorylation at Thr668 is found in AD brains (Chung 2009). Several PK are being proposed as candidates to be responsible for phosphorylation at this residue, including, Cdk5, Cdc2, GSK3, JNK3 and Dyrk1, some of which were shown to be increased in AD brains (Chung 2009). APP phosphorylated at this specific residue is preferentially localized at

neurites of cultured hippocampal neurons while, AICD fragment phosphorylated at Thr668 localizes mainly at the nucleus (Muresan and Muresan 2004). Phosphorylation of APP at Thr668 residue is thought to be relevant to APP neuronal function and processing. Phosphorylation of this residue leads to a conformational change in the cytoplasmic domain, including the Fe65 binding-motif (YENPTY), which may affect the interaction between APP and this protein. Some studies reveal that AICD phosphorylation on Thr668 enhances the formation of a ternary complex between AICD, Fe65 and Tip60 facilitating its translocation to the nucleus. At the nucleus, it may lead to gene transcription. Some of the genes proposed as targets of this regulation, include, KAI, GSK-3β, APP, BACE1, Tip60 and neprilysin (Chang et al. 2006). In the case of GSK-3β, increased expression may follow Tau phosphorylation and hence contribute to NFT formation and neuronal desregulation.

At the APP processing level, Feyt et al. 2007 showed that APP phosphorylation at this residue increases β-cleavage but γ-cleavage is reduced, decreasing Aβ production, indicating that APP phosphorylation also regulate APP processing. Pin-1 was also described as interacting with APP CTF fragment (C99) when phosphorylated at Thr668. This interaction seems to affect γ-cleavage, inducing the Aβ generation (Akiyama et al. 2005).

Among the eight possible phosphorylation sites of APP we can also found the Tyr682. Tyr682 is included in Y682ENPTY687 motif that is a canonical endocytic signal for membrane associated receptors and regulates APP internalization and Aβ formation in vitro. This motif is also important for protein-protein interactions that regulate APP metabolism and signalling in vitro. It has been reported that phosphorylation of APP at Tyr682 represents a "biochemical switch" that drastically changes the APP interactome and the molecular composition of APP complexes (Barbagallo et al. 2010). For instance, phosphorylation at Tyr682 residue abolish the binding of some proteins, such Fe65, Fe65L1, Fe65L2, to APP and create docking sites for others, namely, Pin1, ShcA, SHCB, ShcC, Grb2, Grb7, Nck, Abl, Lyn and Src (Barbagallo et al. 2010). The binding is further increased when APP is also phosphorylated at Thr668 (Tamayev et al. 2009). Shc and Grb2, that are up-regulated in AD brains (Russo et al. 2002), interact with APP but require APP phosphorylation at Tyr682. This could lead to the activation of MAPK pathway, since Shc and AGrb2 are known to link growth factor receptors to signalling pathways, such Ras, MAPK and PI3K, and participate in key processes, such as oncogenic proliferation, neuronal development, cell differentiation and apoptosis (Tamayev et al. 2009; Tarr et al. 2002).

Constitutively active form of the non-receptor tyrosine kinase Abl, overexpression of the nerve growth factor receptor TrkA and Src kinase mediate the phosphorylation of APP at this specific

residue (Barbagallo et al. 2010; Tarr et al. 2002). Abl can also form stable complexes with APP since APP phosphorylation by Abl forms a YENP motif that is recognized and bound by the SH2 domain of Abl itself. The same motif seems to be involved in binding of APP to Shc proteins (Russo et al. 2002). Also, Tyr682 phosphorylation may commit APP to β-secretase cleavage, formation of β-CTF, binding Shc/Grb2 and subsequent signalling activation. β-CTF can also become a γ-secretase substrate for Aβ formation (Schettini et al. 2010).

These phosphorylation events are detected in brains of normal subjects as well in AD brains (Zhou et al. 2004). Studies indicate that Tyr682 is hiperphosphorylated in AD brains (Russo et al. 2001) and it's plausible that excessive phosphorylation of this residue could lead to toxic effects since it plays a role in targeting APP for degradation via secretase, lysossomal or proteosomal pathways (Barbagallo et al. 2010).

2. OBJECTIVES

AD is a complex neurodegenerative disorder characterized neuropathologically by the presence of senile plaques and neurofibrillary tangles, synaptic loss and consequently neurodegeneration. The Aβ peptide, the major constituent of senile plaques, is a key player in AD pathology. Increased Aβ production and aggregation was associated with neurotoxicity, activation of inflammatory response and apoptotic and signaling cascades, therefore contributing to neurodegeneration and gradual cognitive decline. Altered signal transduction is also one of the key aspects in AD pathology. Phosphorylation of proteins is recognized as a fundamental mechanism by which the regulation of key intracellular events is achieved. Several studies have reported abnormal protein kinase and protein phosphatase activities in AD brains as well as abnormal phosphorylation levels of APP and Tau proteins itself. Further, APP interaction with its binding partners is also regulated by phosphorylation, with the phosphorylated or dephosphorylated state of APP enhancing or diminishing the interaction with certain binding proteins.

The main aim of this project was to address how Aβ itself affects protein phosphorylation hence contributing to the understanding of the molecular mechanism triggered by Aβ. Therefore, the following specific aims were to:

- Study Aβ mediated effects on APP phosphorylation;
- Determine the protein phosphatases involved in APP dephosphorylation;
- Evaluate the effect of Aβ on the APP interactome;
- Study the Aβ role on protein phosphorylation;
- Contribute to defining the AD phosphoproteome.

3. METHODS

3.1 Cell Culture

The experiments were carried out in primary rat neuronal cultures. Cerebral cortex and hippocampus were dissected from Wistar Hannover rat embryo at 18th day of gestation and dissociated with trypsin (0.23 or 2.25 mg/ml for cortical and hippocampal cultures, respectively), and deoxyribonuclease I (0.15 or 1.5 mg/ml for cortical or hippocampal cultures, respectively) in Hanks balanced solution (HBSS). Cells were washed with HBSS supplemented with 10% FBS to stop trypsinization, centrifuged at 1000 rpm for 2 minutes, and further washed and centrifuged with HBSS for serum withdraw. Cells were plated onto poli-D-lysine coated dishes at a density of 1,0x10^5 cells/cm^2 and cultured for 10 days in Neurobasal medium (Gibco) supplemented with 2% B27 (final concentration), a serum-free medium combination. The medium was further supplemented with glutamine (0.5 mM), gentamicin (60 µg/ml), and glutamate (25 µM). Cells were maintained in an atmosphere of 5% CO_2 at 37^{o}C and observed in an inverted optical microscope. Five days after plating, 25% of culture medium was replaced with glutamate-free complete Neurobasal medium. After 10 days in culture, cells were used for experimental procedures.

3.2 Experimental Procedures

Cells were incubated with crescent concentrations of aggregated Aβ_{1-42} (American Peptide) for different periods of time. Aβ_{1-42} was reconstituted in H_2O ultrapure (1 mM stock) and aggregated in PBS for 48 hours at 37^{o}C (100 µM aggregated stock). Cells were plated as described in section III. 1. and washed twice with PBS before treatments. Control cultures received PBS vehicle. Cells were incubated with 0.5, 2, 10 and 20 µM Aβ_{1-42} concentrations, for 30 minutes, 3 and 24 hours, in a B27-free Neurobasal medium combination.

Additionally experiments were carried out using PP inhibitors in order to evaluate the PP effects on APP phosphorylation. Cells were exposed to okadaic acid (OA) and cantharidin (Cant) for 30 minutes and 3 hours. Different concentrations of these drugs inhibit different PP as you can see in Table 5.

Table 5. IC_{50} of Ser/Thr protein phosphatase inhibitors (Swingle et al. 2009).

	PP1	PP2A	PP2B	PP4	PP5	PP7
Okadaic Acid (nM)	15-50	0.1-0.3	4000	0.1	3.5	>1000
Cantharidin (nM)	1100	194	>10000	50	600	ND*

*ND – Not determined

3.3 Sample Collection and Immunodetection

After the appropriate treatments, cells lysates were collected in RIPA buffer and sonicated twice during 5 seconds. Samples were stored at -20°C. Protein determination content was perform using BCA assay (see below) and normalized protein samples were electrophoretically separated by 5-20% gradient SDS-PAGE gels (see Section 3.3.2). Separated proteins were transferred onto a nitrocellulose membrane (see Section 3.3.3) followed by immunoblotting for the specific protein (see Section 3.3.3). Detection was carried out using a chemiluminescent method (see Section 3.3.3). The resulting bands were quantified by densitometry (see Section 3.6).

3.3.1 Protein Concentration Determination

For protein quantitation of cell lysates the BCA protein assay (Pierce), based on the use bicinchonic acid (BCA) for the colorimetric detection and quantitation of total protein, was used. This test is based on the capability of proteins to reduce Cu^{2+} to Cu^{+} in an alkaline environment (the biuret reaction). BCA produces a purple colour in the presence of the reduced Cu^{+} ion that results from chelation of two molecules of BCA with one cuprous ion. These soluble complexes exhibit a strong absorbance that can be read at 562 nm.

The quantitative analyses were carried out using 10 µl (for western blotting analysis) and 50 µl (for immunoprecipitation and phospho kit analysis) of the collected cell lysates. The standard curve was prepared by plotting BSA standard absorvance vs BSA concentration, and used to determine the total protein concentration of each sample (Table 6). Both samples and standards were incubated with 1ml of working reagent, which is prepared with 50 parts of reagent A to 1 part of reagent B. All samples were incubated at 37°C during 30 minutes, and cooled to room temperature and immediately measured at 562 nm.

Table 6. Standards used in BCA protein assay method. BSA, Bovine Serum Albumin solution (2mg/ml); WR, Working Reagent.

Standard	BSA (µl)	H_2O (µl)	Protein Mass (µg)
P0	0	50	0
P1	1	49	2
P2	2	48	4
P3	5	45	10
P4	10	40	20
P5	20	30	40
P6	40	10	80

3.3.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method used to separate components of a protein mixture based on their molecular weight and negative charge. The principle of SDS-PAGE relies on the capacity for proteins to migrate through gel pores when placed under an electrical field. The gel percentage and size depend on the molecular weight of the proteins to be separated. As proteins have different electrical charges that affect their mobility, SDS is usually added to protein samples and buffers to confer a negative charge to all proteins, ensuring they will migrate toward the positively charged anode. SDS is also used in combination with a reducing agent (mercaptoethanol) and heat to dissociate proteins before they are loaded on the gel. SDS also breaks up aggregates and non-covalently bound multimers. Gels are composed by 2 phases, the non-restrictive large pore called stacking gel and the resolving gel with a linear progression of acrylamide concentrations (5 to 20%) from top to bottom, resulting in a wider separation range. The gel was prepared and allowed to polymerize at room temperature for 45 minutes. Subsequently, the stacking gel solution was prepared and loaded on the top of the resolving gel, and left to polymerize at room temperature for 30 minutes.

The samples to be run on the gel were boiled in SDS gel loading buffer for 10 minutes to ensure protein desnaturation. Precision plus protein standards Dual Color (BioRad) were used as markers. Proteins were separated electrophoretically (90 mA) for 3-4 hours in a Hoefer electrophoresis system.

3.3.3 WESTERN BLOTTING ANALYSIS

Western blotting is the technique used for detection of specific proteins in complex samples like cell lysates, cell culture supernatants or body fluids. In this technique the proteins separated by electrophoresis are transferred to a solid membrane by an electrophoretic field. This is a fast and efficient procedure and preserves the high-resolution separation of proteins by SDS-PAGE. Once in the membrane, proteins are suitable to detection by total protein staining or labeling the proteins of interest with specific antibodies.

- **Transfer of proteins from the membrane to a solid support**

Proteins were electrophoretic transferred to a nitrocellulose membrane. The gel was placed in contact with a nitrocellulose filter and then sandwiched between Whatman 3MM paper, two porous pad and two plastic supports. The sandwich was immersed in an electrophoretic tank containing transfer buffer. The nitrocellulose filter was placed toward the anode. An electric current of 200 mA was applied for at least 16 hours. After the transfer of the proteins, the membrane was removed from the sandwich and allowed to dry at room temperature.

- **Immunological detection of the immobilized proteins - Chemiluminescent protein detection**

Transferred proteins were probed with specific antibodies (Table 7). Rabbit polyclonal antibody p-APP Thr668 was used to detect APP phosphorylation at Thr668 residue, rabbit polyclonal antibody p-APP Tyr_{682} (corresponding to Tyr_{757} in 770 isoform numbering) was used to detect APP phosphorylation at Tyr682 residue, rabbit polyclonal antibody anti-APP (APP C-terminal) was used to detect full-length APP and mouse monoclonal anti-β-Tubulin was used to detect β-Tubulin.
Membranes were initially soaped in 1x TBS for 5 minutes. Blocking of possible non-specific binding-sites of the primary antibody was performed using 5% (w/v) BSA (for phospho-specific antibodies) or non-fat dry milk (for C-terminal and β-Tubulin antibodies) in 1x TBS-T solution for 4 hours. Subsequently, membranes were incubated with an unlabeled primary antibody direct against the target protein for 4 hours with agitation at room temperature plus overnight at 4°C. Prior to incubation with second antibody, 3 washes (10 minutes each) with 1x TBS-T were performed. Membranes were incubated with second antibody (coupled with horseradish

peroxidase) for two 2 hours. Membranes were additional washed three times with 1x TBS-T before being submitted to the detection method.

The detection method used was ECL or ECL plus, chemiluminescent reagents, depending on the protein detected (Table 7). The ECL (enhanced chemiluminescence) reaction is based on the oxidation of the cyclic diacylhydrazide luminal and ECL Plus is based on the enzymatic generation of acridinium ester, which produces a more sensitive light emission of longer duration than ECL. The membranes were incubated with the working mixture of the chemiluminescent detection reagent. The incubation period was 1 minute for ECL and 5 minutes for ECL plus, at room temperature in the dark room. The membranes were exposed to autoradiography films (Kodak) in an X-ray film cassette. Films were developed and fixed with appropriate solutions (Kodak).

Table 7. Primary and secondary antibodies used to detect the proteins of interest as well as the detection method used for which one.

Protein	First Antibody	Species Reactivity	Secondary Antibody	Detection Method
APP p-Thr668	Polyclonal Thr668 Dilution 1:1000 (Cell Signalling)	Human, mouse, rabbit	Peroxidase 29abelled anti-rabbit Dilution 1:5000 (Amersham Pharmacia)	ECL Plus
APP p-Tyr682	Rabbit Polyclonal anti-APP (phospho Y682) Dilution 1:1000 (Abcam)	Mouse, human, rat	Peroxidase labeled anti-rabbit Dilution 1:5000 (Amersham Pharmacia)	ECL Plus
APP C-terminal	Rabbit anti-APP Dilution 1:500 (Invitrogen)	Human, mouse, pig, rat	Peroxidase labeled anti-rabbit Dilution 1:5000 (Amersham Pharmacia)	ECL
Tubulin	Monoclonal anti-beta-tubulin Dilution 1:5000 (Zymed)	Human, bovine, mouse	Peroxidase labeled anti-mouse Dilution 1:5000 (Amersham Pharmacia)	ECL

3.4 Co-immunoprecipitation

Immunoprecipitation (IP) is a technique in which a target protein, antigen or protein complex (that is bound to our protein of interest) is precipitated from a solution using a specific antibody. Is widely used to estimate molecular weight, identity, quantity and expression levels of a protein of interest and, to study protein-protein interactions. The magnetic Dynabeads are the solid support used in our immunoprecipitation approach. The immunecomplexes attached to Dynabeads are easily removed from the supernadant by magnetic separation. The pure

precipitate can then be eluted from the beads and analyzed by western blotting or mass spectrometry. In this specific case Dynabeads are coupled to Protein G that has different affinity to Ig's of different species.

After the appropriate treatments, cells were washed in 1x PBS and collected with 750µl of lysis buffer – MOPS (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 1 mM NaOrt, 1% Triton X-100) containing protease inhibitors (1 mM PMSF, 3 mM Benzamidine, 5 µM Pepstatin A, 10 µM Leupeptin, 1 mM DTT). The samples were sonicated for 10 seconds. After BCA protein quantification (see Section 3.3.1.), normalized lysates were pre-cleared with 15 µl DynaBeads Protein G, for 1 hour at 4°C with agitation. DynaBeads Protein G were previously washed twice with 3% BSA/1x PBS. Subsequently the supernadant was transferred to a new microtube that contains 40 µL DynaBeads conjugated with the specific primary antibodies (APP p-Thr668 and C-Terminal, both at dilution of 1:100) and incubate overnight at 4°C with agitation. Further, the supernadant was transferred to a new microtube and the DynaBeads washed three times with 3% BSA/1x PBS and three times with 1x PBS (10 minutes each, at 4°C with agitation). Finally, DynaBeads were ressuspended in 45 µL of 1x Loading Buffer and boiled at 70°C for 10 min. Immunoprecipitates were frozen at -20°C and shipped to Kinexus to Mass Spectrometry Services PIMS (Protein ID by mass spectrometry).

3.5 Phosphoprotein Extraction

To achieve phosphoprotein extraction, a Phosphoprotein Enrichment Kit (Clontech) based on affinity chromatography was used. This technique separates proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatographic matrix. It can be used as a purification step (like in this case), is high selectivity and gives high resolution. In this case, phosphate groups of protein selectively bounds to the phosphate metal affinity chromatography resin and non-phosphorylated proteins and contaminants pass through the resin. Then an elution buffer is added to the resin and phosphorylated proteins elutes from the resin.

After treatment with aggregated Aβ (10 µM), cells were washed twice in 1x PBS and collected with 7 ml of 1x PBS and centrifuged twice, 5 and 2 minutes, at 500 g. Extraction/Loading Buffer with sodium fluoride (final concentration of 10 mM) was added to the cell pellet (30 µl for each mg of pellet). The cell lysate was transferred to a new microtube and centrifuge at 10000 g for 20 minutes at 4°C to remove insoluble cellular debris. The supernadant was transferred to a new

microtube and 50 µl of the sample was used to perform the BCA assay. The cellular extract is loaded on a Phosphoprotein Affinity Column. The column was closed and gently agitates at 4°C for 20 min on a platform shaker to allow the phosphorylated proteins to bind to the column. The PMAC Resin is highly selective for the phosphates on the proteins, allowing other proteins and contaminants to pass through in the flowthrough and wash. The column was stand in the upright position to allow the resin to settle out of suspension. Then, the non-adsorbed material, that contains the non-phosphorylated proteins, was left to flow through and collected. The column was washed 4 times by adding 5 ml of Extraction/Loading Buffer.

Then the phosphorylated proteins are eluted from the column with Buffer B (5 ml). One ml of Elution Buffer was added at a time to the collumn and the fraction collected on ice. Five fractions of phosphorylated proteins were collected. A BCA assay (see Section 3.3.1.) was performed with 50 µl of each fraction. Fractions 2 were the most phospho-enriched fractions and were therefore analyzed by mass spectrometry.

3.6 Quantitative analysis

Quantitative analysis was performed using densitometric scanning using a BioRad system.

4. RESULTS

4.1 Aβ effects on APP phosphorylation

This work was aimed at studying the effects of Aβ on APP phosphorylation, specifically, at APP Thr668 and Tyr682 residues. In order to accomplish this aim, rat cortical primary cultures were used and incubated with different concentrations of Aβ for different incubation periods, as explained in Section 3.2.

We first evaluated Aβ effects on holo APP ($_{Holo}$APP) levels. Experiments carried out showed that Aβ exposure for 30 minutes had no effect on APP levels, as detected using an APP C-terminal antibody (Figure 9 A). However, incubation for 24 hours led to a decrease in $_{Holo}$APP at the higher Aβ concentrations. These observations are in agreement with previous work from our laboratory (Henriques et al. 2009).

After analyzing Aβ mediated effects on $_{Holo}$APP content and in order to address the Aβ role on APP phosphorylation, cell lysates were probed with phospho-specific antibodies. In particular, Aβ effects on APP phosphorylation at Thr668 and Tyr682 were determined. Data is expressed normalized for $_{Holo}$APP and β-Tubulin content.

Relative to APP phosphorylation at the Thr668 residue, the phosphorylation pattern is different depending of the incubation period. After 30 minutes exposure to Aβ, cortical primary cultures exhibit an initial increase in phosphorylation for the lowest concentrations, reaching a maximum at 10 µM, but the phosphorylation level is diminished at 20 µM. Overall, longer periods of Aβ incubation led to a decrease in the phosphorylation pattern of APP at this residue, in particular at the highest Aβ concentration, upon 24 hours exposure (Figure 9 B).

For APP phosphorylation at the Tyr682 residue, this showed a different phosphorylation pattern in comparison to Thr668. In response to Aβ incubation for 30 minutes we could observe an increase in the phosphorylation levels, maximum with Aβ 20 µM. Almost all Aβ concentrations and incubations periods render an increase in phosphorylation levels of APP at Tyr682 (Figure 9 C), although the 3 hours time point was less evident. At 24 hours the phosphorylations APP levels also increased at this residue and although there was a decrease at the higher concentrations, this remained above control levels. The oscillations observed are likely to reflect the dynamics typical of protein phosphorylation systems and deserve further investigation. Similar data was obtained for primary hippocampal cultures (data not shown).

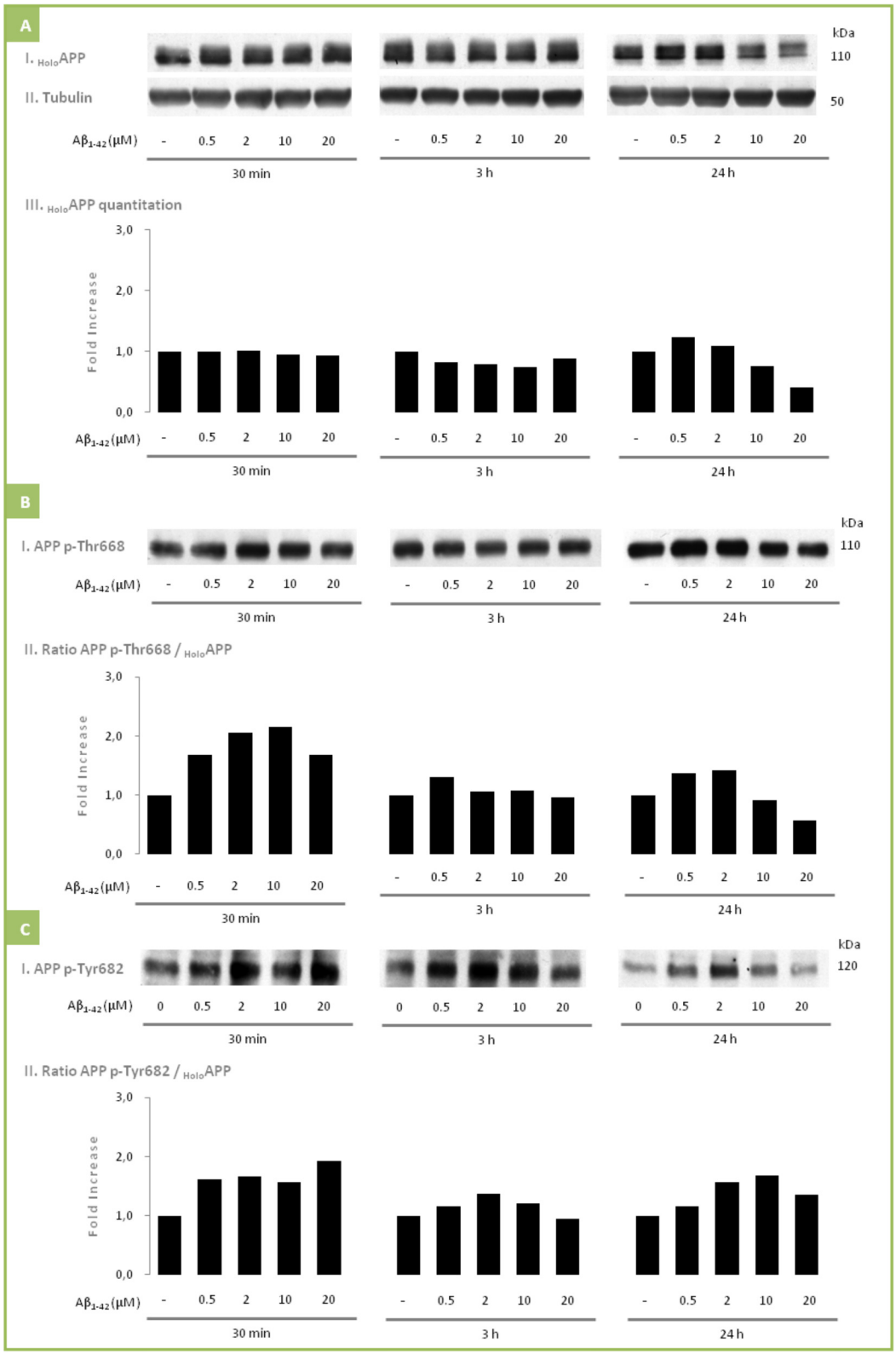

Figure 9. Aβ effects on APP phosphorylation at Thr668 and Tyr682 residues. Cortical primary cultures were incubated for 30 minutes, 3 and 24 hours with aggregated $A\beta_{1-42}$ (0.5, 2, 10 and 20μM). **A.** Cell lysates were collected and analyzed by Western blot with C-Terminal (I) and β-Tubulin (II) antibodies and $_{Holo}$APP were quantified (III) **B.** Cell lysates were also analyzed with phospho APP Thr668 (I) and quantitation of APP phosphorylated at Thr668, with values normalized to $_{Holo}$APP content (II). **C.** Analysis of cell lysates with phospho APP Tyr682 (I) were also analyzed and APP phosphorylation level at Tyr682 was quantified, with values normalized to $_{Holo}$APP content (II). Tubulin was used as a control protein. Data were obtained from triplicate experiments.

4.2 Protein phosphatases involved in APP dephosphorylation

In order to evaluate the PP involved in APP dephosphorylation at Thr668 residue, two PP inhibitors, okadaic acid and cantharidin, were used. Okadaic acid (OA) has as primary targets PP1 and PP2A while cantharidin has PP2A as its primary target. However, different concentrations of these inhibitors are able to inhibit different PP's besides PP1 and PP2A as indicated in Table 5 (Section 3.2). For these studies cortical primary cultures were incubated with different concentrations of these inhibitors in order to discriminate the possible PP involved in this APP phosphorylation process. Although the inhibitors here used are not directed at Tyr, APP Tyr682 phosphorylation was nonetheless monitored, given that protein phosphorylation occurs as a sequence of events, and crosstalk between Ser/Thr and Tyr phosphorylation does occur.

Cortical primary cultures were treated with OA for 30 minutes and 3 hours and a decrease in $_{Holo}$APP levels at higher OA concentrations was detected. These values were taken in consideration when determining the PP inhibitors effects on APP Thr668 and Tyr682 phosphorylation levels.

After 30 minutes of incubation, phosphorylation levels of APP at the Thr668 residue remained unchanged until the concentration of 500 nM. At this concentration, where PP1 is inhibited and at 5000 nM, the phosphorylation level of APP doubled when compared to control (Figure 10 B). An additionally increase was observed with 5000 nM of OA where PP2B is inhibited. The response was also observed after 3 hours treatment, where the two highest concentrations led to an increase in phosphorylation of APP at Thr668. At 0.1 and 0.25 nM, where PP4 and PP2A are respectively inhibited, no differences could be observed in the phosphorylation level of APP.

The cell lysates were also probed with the phospho APP Tyr682 antibody. OA is an inhibitor of serine/threonine phosphatases, therefore it was not expected a different effect of PP inhibition in this residue. However, variations in the phosphorylation level were detected in particular at 3 hours incubation, where we observed a decrease in the phosphorylation rate of Tyr682 (Figure 10 C). Additionally, we could also detect an alteration in the migration pattern at the OA concentrations of 50, 500 and 5000 nM. This set of results clearly revealed that Tyr682 phosphorylation also involves other Ser/Thr phosphorylation events, which must be further addressed. This is particularly evident given that the effect is augmented at the 3 hour time point; refelective of the accumulation of other Ser/Thr dependent reactions, where key dephosphorylation events have been inhibited.

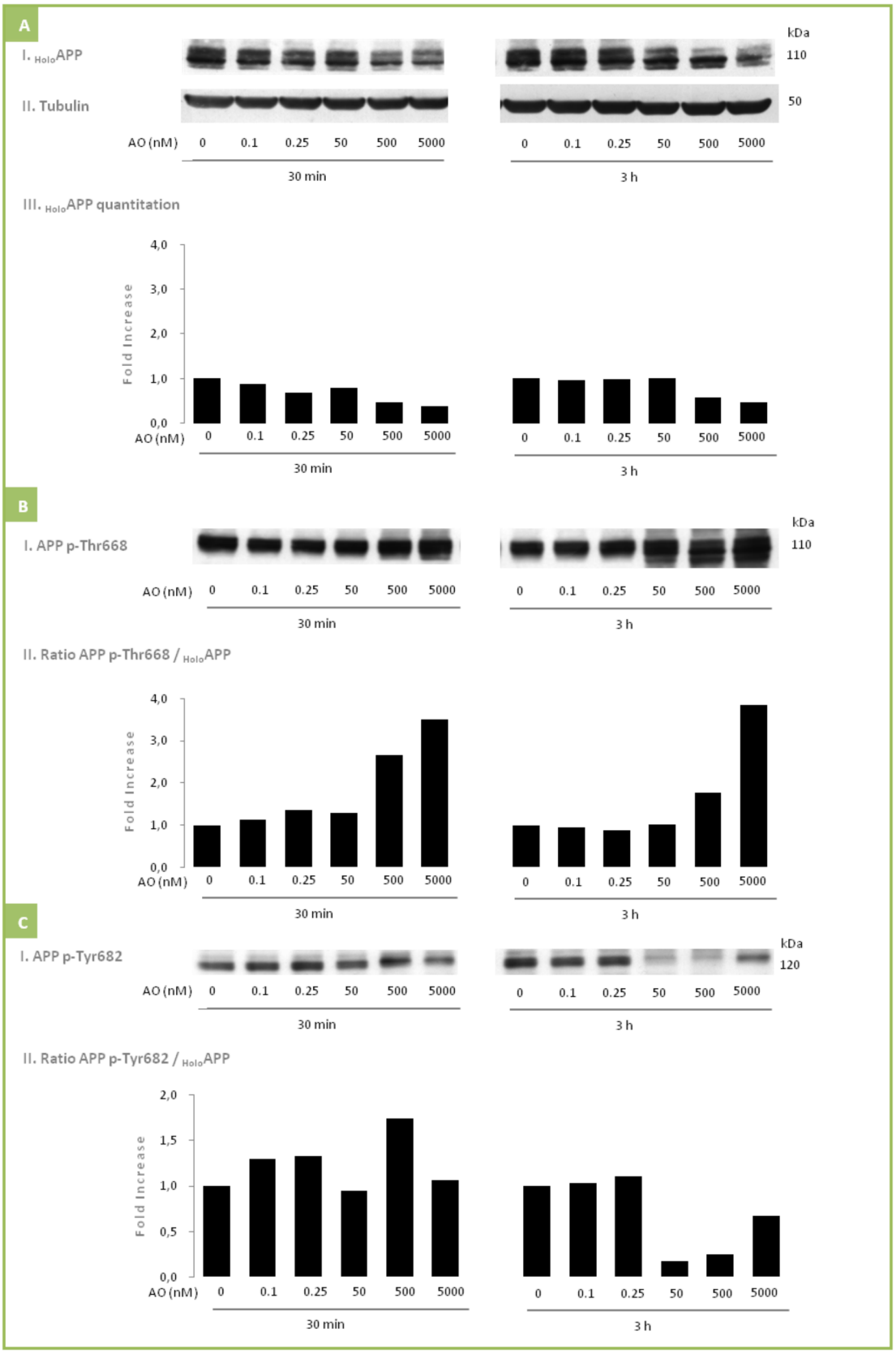

Figure 10. Okadaic acid effects on APP phosphorylation at Thr668 and Tyr682. Cortical primary cultures were incubated for 30 minutes and 3 hours with different concentrations of okadaic acid (0.1, 0.25, 50, 500 and 5000 nM). **A.** Cell lysates were collected and analyzed by Western blot with C-terminal antibody (I) and $_{Holo}$APP content was quantified (II) **B.** Cell lysates probed with phospho APP Thr668 antibody (I) and ratio with $_{Holo}$APP content (II) **C.** Cell lysates probed with phospho APP Tyr682 antibody (I) and ratio with $_{Holo}$APP content (II). Tubulin was used as a control protein. Data were obtained from duplicate experiments.

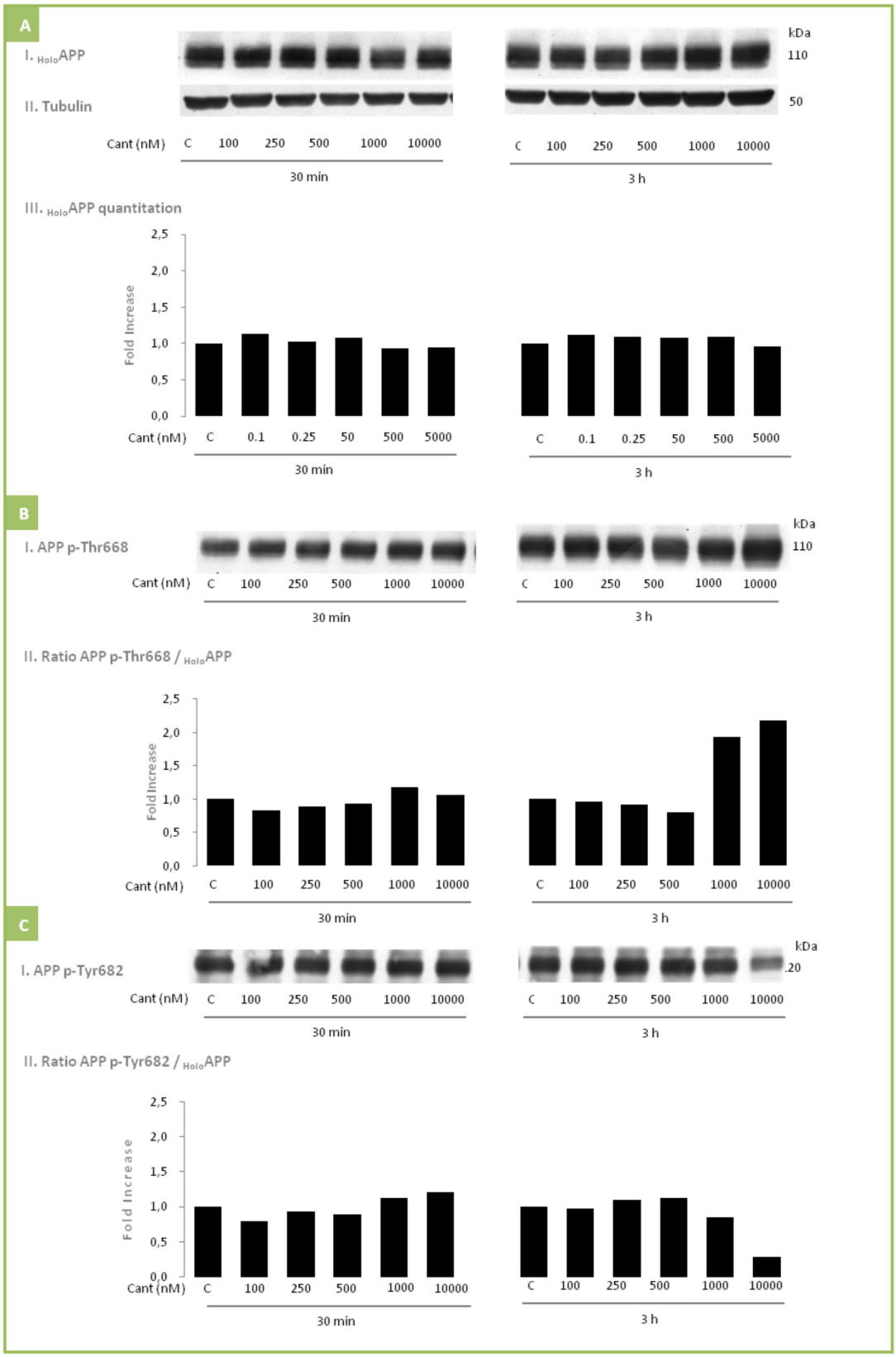

Figure 11. Cantharidin effects on APP phosphorylation at Thr668 and Tyr682. Cortical primary cultures were incubated for 30 minutes and 3 hours with different concentrations of cantharidin (Cant) (100, 250, 500, 1000 and 10000 nM). **A.** Cell lysates were collected and analyzed by Western blot with C-terminal antibody (I) and $_{Holo}$APP content was quantified (II) **B.** Cell lysates probed with phospho APP Thr668 antibody (I) and ratio with $_{Holo}$APP content (II) **C.** Cell lysates probed with phospho APP Tyr682 antibody (I) and ratio with $_{Holo}$APP content (II). Tubulin was used as a control protein. Data were obtained from duplicate experiments.

When using cantharidin, similar results were obtained in terms of APP phosphorylation at Thr668, particularly for the 3 hour time point. An increase in the phosphorylation level was observed at 1000 and 10000 nM where PP1 and PP2B, are inhibited respectively (Figure 11 B). A slightly increase is also observed with 500 nM, where PP2A is inhibited however, when compared with the increase observed when PP1 and PP2B are inhibited, this only increases marginally. Clearly this will now be further investigated in order to validate the results.

In the case of APP phosphorylation at Tyr682, some small fluctuations were evident. The most marked response was a decrease for 3 hours incubation at the highest concentrations (Figure 11 C).

4.3 AD related protein interactome

This section addressed how the AD related interactome could be affected by Aβ and OA. The approach used was to study the various proteins immunoprecipitating with APP under the different incubation conditions by MS, or by analysing the fractions enriched for phosphorylated proteins. Of note, the laboratory has previously shown that Aβ can inhibit PP1 (Vintém et al 2009).

4.3.1 Analysis following immunoprecipitate pull-down

As a result of various studies several APP binding proteins have thus far been identified. In order to evaluate how Aβ affects the APP phospho-dependent interactions, co-immunoprecipitation assays were performed as described in Section 3.4. Co-immunoprecipitation was carried-out using APP Thr668 phospho-specific and the C-terminal antibodies upon Aβ and OA treatment for 3 hours. Samples were normalized for protein content and separated by SDS-page and stained with Comassie Blue. Bands 1-9 (Figure 12) were extracted and are presenlty being analyzed by mass spectrometry (kinexus).

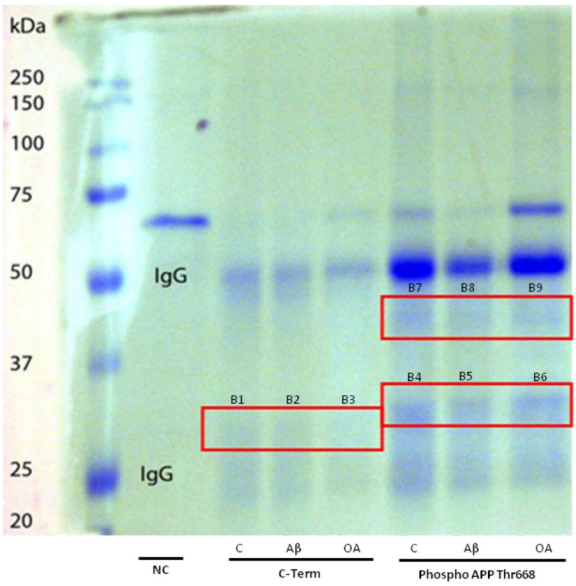

Figure 12. Protein composition of the immunoprecipitates. Polyacrylamide gel stained with Coomassie Blue. At 50 and 25 kDa are the bands of the heavy and light chains, respectively, of the antibodies used in the immunoprecipitation. The bands to be analyzed are indicated in the red boxes. NC- Negative Control; C- Control immunoprecipitated with C-Terminal antibody; Aβ - Cells treated with 10 µM of aggregated; OA - Cells treated with 0.25 µM of okadaic acid; B1-B3 – These bands were lightly stained and diffuse, so in an attempt to give a positive MS ID, they were collected and will be analyzed as one; B4-B9 – Bands will be analyzed separately. At 50 and 25 kDa are the bands of the heavy and light chains, respectively, of the antibodies used in the immunoprecipitation.

4.3.2 Analysis of phosphorylation enriched samples

Cortical primary neuronal cultures were treated with 10 µM of aggregated $A\beta_{1-42}$. After treatment, cellular extracts were loaded on a Phosphoprotein Affinity Column, following the Phosphoprotein Enrichment Kit (Clontech) instructions (Section 3.5). As a result of this procedure two enriched fractions were obtained: one containing the phosphorylated proteins and the other containing the non-phosphorylated proteins. The fraction containing the phosphorylated proteins was analyzed by mass spectrometry in order to evaluate alterations in the phosphoproteome under control and upon Aβ treatment. The obtained peptides were identified by search in a mouse cDNA database and resulted in the identification of 1008 proteins.

Analysis of the functional protein groups in the different conditions showed that Aβ treatment led to an increase in the following groups; enzymes/metabolic (21,2 vs 18,0%), gene transcription/nuclear proteins (23,2 vs 21,4%), kinases/phosphatases (9,9 vs 8,7%) and stress/apoptotic group (6,8 vs 2,5%). In the cell cycle/differentiation and synaptic protein groups we detected a phosphoprotein decrease (Figure 13 A).

When looking specifically at the different proteins that appear in each condition (Figure 13 B), under control conditions the two groups of proteins with higher percentage are the gene transcription/nuclear proteins (23,0%) and the cytoskeletal proteins (21,3%), followed by the subcellular compartment proteins (18,0%), the enzymes/metabolic (9,8%) and cell cycle/differentiation (9,8%). These percentages varied in the Aβ exposure condition. The four main groups of proteins include the gene transcription/nuclear proteins (29,7%) and the enzymes/metabolic (25,3%), cytoskeletal proteins (18,7%) and kinases/phosphatases (13,2%). In Aβ condition we could detect a decrease in the phospho cytoskeletal proteins when compared to control (18,7% vs 21, 3%).

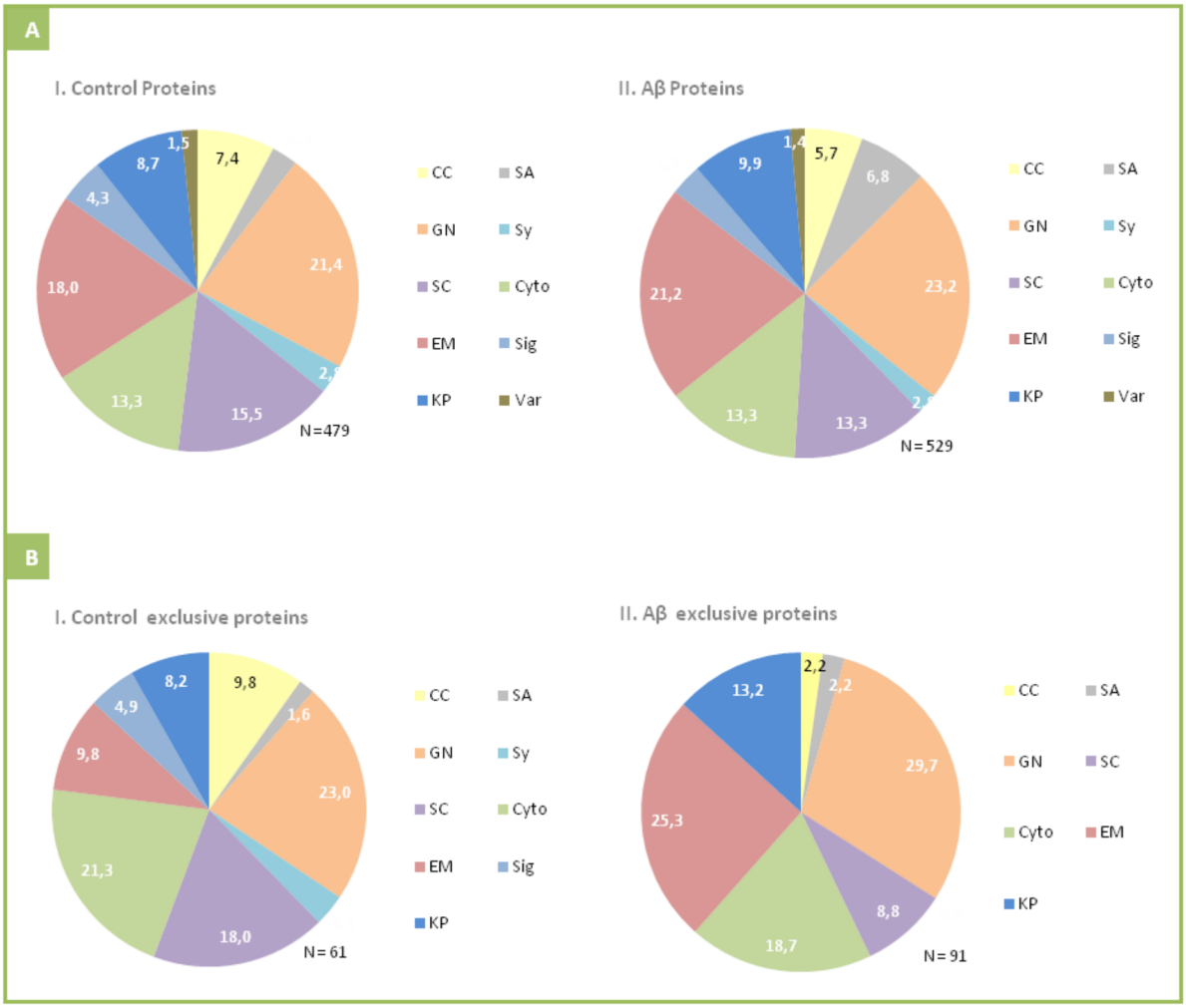

Figure 13. A. Aβ effects on the APP phosphoproteome. As global analysis, phosphoproteins extracted were grouped by their function in each condition, control (I) vs Aβ (II). **B.** Exclusive phosphoproteome. Analysis of specific phosphoproteins for each condition (control (I) and in Aβ response (II). CC – Cell cycle/differentiation proteins; SA – Stress and apoptotic proteins; GN - Gene transcription/nuclear proteins, Sy – Synaptic proteins; SC – Subcellular compartment organelles complexes; Cyto - cytoskeletal proteins; EM - enzymes/metabolic proteins; Sig – Signalling; KP – Protein kinases/phosphatases.

5. Discussion & Conclusion

5.1 Aβ Effects on APP phosphorylation

Protein phosphorylation is a key mechanism involved in many cellular processes and signaling cascades. Also, abnormal protein phosphorylation has been associated with many neurodegenerative disorders including AD. One of the mechanisms that regulate APP function and processing is protein phosphorylation. APP is a phospho-specific protein that exhibits two potential phosphorylation sites in its ectodomain and eight potential phosphorylation sites in the AICD region. Additionally, due to its ability to trigger a set of biochemical and cellular alterations that lead to neurodegeneration, Aβ has been described as a key player in AD pathogenesis. One of the mechanisms by which Aβ may contribute to AD pathology is the induction of abnormal protein phosphorylation. In fact, several studies have reported that Aβ may affect the activity of several kinases (Chung 2009) and also PP (Chung 2009; Vintém et al. 2008). In this work we aim to further investigate Aβ effects on protein phosphorylation including APP itself. In order to evaluate Aβ effects on APP phosphorylation, two APP residues (APP Thr668 and APP Tyr682) were analyzed. In response to low concentrations of aggregated $A\beta_{1-42}$ we could detect an increase in the phosphorylation of both residues. These results are consistent with the "*in vivo*" observations, that both residues are abnormally phosphorylated in AD brains (Barbagallo et al. 2010). The highest Aβ concentrations led to a decrease in phosphorylation of APP at Thr668. Physiologically, the amounts of Aβ are in the low nanomolar range and values in micromolar range are considered pathological and where the aggregation is spontaneous. This concentration gap spans three to four orders of magnitude (Hu et al. 2009). Therefore, at the higher concentrations, other intracellular events may be occurring, e.g. apoptotic events. It has been reported that Aβ potentiates the activity of some PK namely GSK-3β, Cdk5 and c-Abl (Hooper et al. 2008; Resende et al. 2008; Town et al. 2002; Alvarez et al. 2004). The first two are involved in APP phosphorylation at Thr668 and the last one in phosphorylation at Tyr682 residue. Potentially the effects of Aβ on these proteins may contribute towards the increased phosphorylation levels observed.

APP phosphorylation at these specific residues modulates the interactions of APP with other proteins. For instance, the phosphorylation of APP at Thr668 is essential for its binding to Fe65 and to form a complex which can be translocated to the nucleus and interfere with gene transcription. The candidates genes are: KAI, GSK-3β, APP, BACE1, Tip60 and neprilysin (Chang et al. 2006). GSK-3β is one of the PK involved in Thr668 phosphorylation and also in Tau phosphorylation. Expression of this kinase may affect both APP and Tau phosphorylation,

contributing to the abnormal APP function and NFT formation. APP phosphorylation at this specific residue also regulates APP processing resulting in increased Aβ production. This finding suggests that Aβ production and Thr668 phosphorylation are linked (Shin et al. 2007). Hence Aβ-induced abnormal APP Thr668 phosphorylation is likely to contribute to neural death.

Tyr682 is within a docking site for interaction with cytosolic proteins that regulate APP metabolism and signaling. For example, normal Aβ generation and secretion are dependent upon Tyr682 "*in vitro*" (Barbagallo et al. 2010). However, physiological functions of Tyr682 are unknown. Phosphorylation of APP at Tyr682 is considered to produce a shift in the APP interactome, since it can inhibit the interaction with some proteins (like Fe65) and potentiate the interaction with others. Additionally, there are some proteins, namely Shc and Grb2, that only interact with APP when it is phosphorylated at this specific residue. This interaction can be potentiated by phosphorylation of APP at the Thr668 residue. Therefore, the Aβ effect on APP Thr668 phosphorylation may also affect APP interactions at the Tyr682 docking site. The amounts of these two adaptors proteins, Shc and Grb2, are increased in AD brains suggesting a pathogenic correlation between increased Tyr682 phosphorylation levels and these two adaptors proteins (Russo et al. 2002). It is also possible that Tyr682 phosphorylation may play a role in targeting APP for degradation via secretases, lysosomal or proteasomal pathways, which could explain the absence of the APP Tyr682 phosphorylation in normal brains (Barbagallo et al. 2010).

5.2 Protein phosphatases involved in APP dephosphorylation

Although many studies have been directed at the identification of the PK involved in APP phosphorylation little is known about the PP's involved in APP dephosphorylation. In order to identify the PP involved in APP Thr668 dephosphorylation two PP inhibitors were used. Data obtained suggests that PP1 is the major PP involved in APP Thr668 dephosphorylation. When using concentrations of the inhibitors (okadaic acid and cantharidin) that specifically inhibit PP1, APP Thr668 phosphorylation levels increase. As previously reported by our group (Vintém at al. 2008), Aβ may directly affect the activity of PP1 and therefore contributing to the increased APP Thr668 phosphorylation level.

Since Tyr682 is an important residue, that functions as a "biochemical switch" in the APP interactome, we also studied the effects of PP inhibition on the phosphorylation of this residue. The data obtained does not reflect a direct effect of PP inhibition in this residue since these

inhibitors only affect serine/threonine residues. Nonetheless we could detect alterations in the phosphorylation level of this residue, both with okadaic acid and cantharidin treatment. The results here presented show that PP1 inhibition leads to a decrease in Tyr682 phosphorylation levels. These data suggests that PP exert an indirect effect on the phosphorylation of Tyr residues. This effect is undoubtedly mediated by PK and PP and the signalling cascades involved deserve further investigation.

5.3 Aβ role on APP interactome

The data here presented as well as other published results, permit the affirmation that the APP interactome is shaped by its phosphorylation state. Some of the APP interacting proteins have already been described (Table 2, Section 1.2.5) and the interactions of APP with these proteins are modulated by the phosphorylation state in specific residues, as mentioned before. Thus we aimed to evaluate how Aβ affects the APP interactome when the latter protein is phosphorylated or not at Thr668. Cells were submitted to different treatments and immunoprecipitations were carried out using APP C-Terminal antibody (immunoprecipitated $_{Holo}$APP) and phospho APP Thr668 antibody (immunoprecipitated APP phosphorylated at Thr668 residue). Through the analysis of SDS-PAGE gel stained with Coomassie Blue some differences are evident in the protein pattern under the different conditions. This indicates that both Aβ and APP phosphorylation at this residue in fact modulates the APP interactome. The treatment with okadaic acid and consequently the alteration in the phosphorylation state of APP also seems to influence de APP interactions. The extracted bands are now being analyzed by mass spectrometry.

5.4 Aβ effects on protein phosphorylation

Phosphoproteins were extracted to determine Aβ effects on the holo phosphoproteome. We could observe that Aβ treatment lead to a general increase in the phosphoproteome (479 vs 529 proteins). However, some of the proteins present in each condition appeared uncharacterized and were therefore excluded for subsequent functional analysis.

Abnormal protein phosphorylation is a common feature of many proteins in AD, and as discussed above Aβ affects relevant phosphorylation events. Therefore all the data here presented indicates that Aβ may play a key role in different intracellular processes and signaling cascades.

From the different and distinct group of proteins obtained, we could determine that upon Aβ treatment there was an increase in the percentage of proteins involved in gene transcription plus nuclear functions (GN) and in the group of kinases and phosphatases (KP). Abnormal protein phosphorylation is a common feature of many proteins in AD. Therefore these data are consistent with the increased percentage of the group KP. Additionally, we could also observe an increase in the stress and apoptotic proteins, which is in agreement with well known neurotoxic and apoptotic effects of Aβ.

When looking at the proteins exclusive of each condition, the Aβ related proteins show a decrease in the cytosqueletal protein group. Previous studies by Henriques et al. 2009 have shown an Aβ effect on different cytoskeletal proteins. Abnormal cytoskeletal alterations have been associated with altered protein transport and cytoskeletal dynamics which are fundamental processes for maintaining neuronal viability. All these proteins will now be analysed according to their putative function and relevance to AD pathology.

5.5 Final Conclusions

- At low concentrations Aβ potentiates the phosphorylation state of APP (Thr668 and Tyr682 residues) therefore potentially contributing to abnormal APP processing, interactions and function.

- PP1 is the major protein involved in APP Thr668 dephosphorylation. Aβ was shown to directly affect the activity of this PP, suggesting that it can contribute to the increase in the phosphorylation state of APP, also observed in AD brains.

- Both Aβ and phosphorylation at APP Thr668 may influence APP-protein interactions and hence contribute to abnormal APP function, processing and a potential increased Aβ production.

- Different phosphoproteins could be identified in response to Aβ exposure. The understanding of the functional relevance of these proteins in AD pathology may reveal novel physiological targets for therapeutic intervention.

- Aβ was shown to play a key role in abnormal protein phosphorylation, potentially leading to abnormal signalling cascades that may culminate in neuronal death and neurodegeneration.

- This work allowed for a better understanding of some of the molecular alterations induced by Aβ that may contribute to the disease progression.

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ANNEXES

Below is listed the equipment used and composition of buffers for the different techniques applied.

Cell Culture and experimental models

Equipment

- Hera cell CO2 incubator (Heraeus)
- Safety cabinet Hera safe (Haraeus)
- Inverted optical microscope (LEICA)
- Hemacytometer (Sigma)
- Sonicator (U200S (IKA)
- Bath SBB6 (Grant)

Reagents

- Complete Neurobasal medium (Cortical primary cultures)

  This serum-free medium (Neurobasal; Gibco) is supplemented with:

  - 2% B27 supplement (Gibco)
  - 0.5 mM L-glutamine
  - 60 µg/ml Gentamicine (Gibco)
  - 0,001% Phenol Red (Sigma-Aldrich)

  Adjust to pH 7.4. Sterilize by filtering through a 0.2 µm filter and store at 4°C.

- Complete Neurobasal medium (Hippocampal primary cultures)

  This serum-free medium (Neurobasal; Gibco) is supplemented with:

  - 2% B27 supplement (Gibco)
  - 0.5 mM L-glutamine
  - 25 µM L-glutamate (Gibco)
  - 60 µg/ml Gentamicine (Gibco)
  - 0,001% Phenol Red (Sigma-Aldrich)

  Adjust to pH 7.4. Sterilize by filtering through a 0.2 µm filter and store at 4°C.

- Hank's balanced salt solution (HBSS)

  This salt solution is prepared with deionised H2O. Final Composition:

  - 137 mM NaCl
  - 5.36 mM KCl
  - 0.44 mM KH2PO4
  - 0.34 mM Na2HPO42H2O
  - 4.16 mM NaHCO3
  - 5 mM Glucose
  - 1 mM Sodium pyruvate
  - 10 mM HEPES

  Adjust to pH 7.4. Sterilize by filtering through a 0.2 µm filter and store at 4oC.

- Poly-D-lysine stock (Sigma-Aldrich)

  To a final volume of 10 ml at 10mg/ml, dissolve in deionised H_2O 100 mg of poly-D-lysine. To prepare the poli-D-Lysine solution dilute 1 ml of the 10 mg/ml poly-D-lysine stock solution in borate buffer.

- Borate buffer

  To a final volume of 1 L, dissolve in deionised H_2O 9.28 g of boric acid (Sigma-Aldrich). Adjust to pH 8.2, sterilize by filtering through a 0.2 µM filter, and store at 4^{o}C.

- PBS (1x)

  For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H_2O. Final composition:

  - 8 mM Sodium Phosphate
  - 2 mM Potassium Phosphate
  - 140 mM Sodium Chloride
  - 10 mM Potassim Chloride

  Sterilize by filtering through a 0.2 µm filter and store at 4oC.

- RIPA buffer (Sigma-Aldrich)

  To 6.5 ml of RIPA buffer add:

  - 40.3 µL NaF
  - 65 µL NaOrt
  - 65 µL Protease inhibitor cocktail (Sigma-Aldrich)

- $A\beta_{1-42}$ (American Peptide)

- Okadaic Acid (Calbiochem)

Protein Content Determination

Equipment

- Spectrophotometer Cary 50 (Varian)

Reagents

- BCA assay kit (Pierce, Rockford, IL)
- Bovine Serum Albumin (BSA) (Pierce)
- Working Reagent (50 Reagent A : 1 Reagent B)

  Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,2N sodium hydroxide.
  Reagente B: 4% cupric sulfate.

SDS-PAGE

Equipment

- Electrophoresis system (Hoefer SE600 vertical unit)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

Reagents

- Acrylamide stock mixture (30% acrylamid, 0.8% Bisacrylamide)

  To 70 ml of deionised H_2O add:

  - 29.2 g Acrylamide
  - 0.8 g Bisacrylamide

  Mix until the solute has dissolved. Adjust the volume to 100 ml with deionised water. Filter through a 0.2 µm filter and store at 4°C.

- Stacking gel and resolving gel

	Stacking Gel	Resolving gel	
	3,5%	5%	20%
H_2O	13.2 ml	17.4 ml	2,2 ml
Acrylamide stock mixture	2.4 ml	5 ml	20 ml
UGB (5x)	4.0 ml	--	--
LGB (4X)	--	7.5 ml	7.5 ml
10% APS	200 µl	150 µl	150 µl
10% SDS	200 µl	--	--
TEMED	20 µl	15 µl	15 µl

- UGB (Upper gel buffer) (5x)

  To 900 ml of deionised H_2O add:

  - 75.69 g Tris

  Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1 L with deionised H_2O.

- LGB (Lower gel buffer) (4x)

  To 900 ml of deionised H_2O add:

  - 181.65 g Tris
  - 4 g SDS

  Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with deionised H_2O.

- APS (Ammonium Persulfate) 10%

  In 10 ml of deionised H_2O dissolve 1 g of APS. Note: prepare fresh before use.

- SDS (Sodium Dodecilsulfate) 10%

  In 10 ml of deionised H_2O dissolve 1 g of SDS.

- Loading Gel Buffer (4x)

  - 2.5 mL (250 mM) Tris solution (pH 6.8) 1 M
  - 0.8 g (8%) SDS
  - 4 ml (40%) Glicerol
  - 2 ml (2%) Beta-Mercaptoetanol
  - 1 mg (0.01%) Bromofenol blue

  Adjust the volume to 10 ml with deionised H_2O. Store in darkness at room temperature.

- Tris 1 M (pH 6.8) solution

  To 150 ml of deionised H_2O add 30.3 g Tris base. Adjust the pH to 6.8 and adjust the final volume to 250 ml.

- 10x Running Buffer
  - 30.3 g (250 mM) Tris
  - 144.2 g (2.5 M) Glycine
  - 10 g (1%) SDS

  Dissolve in deionised H_2O, adjust the pH to 8.3 and adjust the volume to 1 L.

WESTERN-BLOTTING

Equipment

- Transphor Electrophoresis unit (Hofer TE 42)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

Reagents

- 1x Transfer Buffer
  - 3.03 g (25 mM) Tris
  - 14.41 g (192 mM) Glycine

  Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionised H_2O. Just prior to use add 200 ml of methanol (20%).

IMMUNOBLOTTING

- 10x TBS (Tris buffered saline)
  - 12.11 g (10 mM) Tris
  - 87.66 g (150 mM) NaCl

  Adjust the pH to 8.0 with HCl and adjust the volume to 1L with deionised H_2O.

- 10x TBS-T (TBS+Tween)
  - 12.11 g (10 mM) Tris
  - 87.66 g (150 mM) NaCl
  - 5 ml (0.05%) Tween 20

  Adjust the pH to 8.0 with HCl and adjust the volume to 1L with deionised H_2O.

- Blocking Solution

  5% of nonfat dried milk or BSA (Bovine Serum Albumin, Merck) in 1x TBS-T.

- ECL or ECL Plus Kits (Amersham Biosciences)

- Developer and fixer Solution (Sigma)

- Membranes Stripping Solution
  - 3.76 g (62.5 mM) Tris-HCl (pH 6.7)
  - 10 g (2%) SDS
  - 3.5 ml (100 mM) Beta-mercaptoetanol

  Dissolve Tris and SDS in deionised H_2O and adjust with HCl to pH 6.7. Add the mercaptoethanol and adjust volume to 500 ml.

IMMUNOPRECIPITATION

- Lysis Buffer
  - 300 µl (20 mM) MOPS 2M (pH 7.0)
  - 240 µl (2 mM) EGTA 250 mM
  - 600 µl (5 mM) EDTA 250 mM
  - 900 µl (30 mM) NaF 50 mg/ml
  - 300 µl (1 mM) NaOrt 100 mM
  - 300 µl (1%) Triton X-100 100%

- Lysis Buffer + Protease Inhibitors

  Add to 28819µl of lysis buffer the following quantities for a final volume of 30 mL:
  - 300 µl (1 mM) PMSF 100 mM
  - 450 µl (3 mM) Benzamidine 200 mM
  - 102 µl (5 µM) Pepstatin A 1mg/ml
  - 28,56 µl (10 µM) Leupeptin 5 mg/ml
  - 300 µl (1mM) Dithiothreitol (DTT)* 0.1 M

  * prepare fresh before use. Add 0.0154 g of DTT to 1ml of deionised H_2O.

- Blocking solution

  Add 0.6 g of Bovine Serum Albumine (BSA) to 20 ml of PBS 1x

PHOSPHOPROTEIN EXTRACTION

Reagent

Phosphoprotein Enrichment Kit Talon PMAC (Clontech)

QUANTITATIVE ANALYSIS

Equipment

GS-710 calibrated imaging densitometer (Bio-Rad)